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ORIGINAL PAPER

Effects of carbon sources and feeding strategies on heparosan production by *Escherichia coli* K5

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Abstract This work aimed to develop an optimal carbon source feeding strategy to achieve maximal production of heparosan as a precursor of bioengineered heparin by *Escherichia coli* K5. Glycerol gave higher heparosan titer and productivity compared to glucose. The maximum heparosan production (187 mg/L) and heparosan productivity (5.19 mg/L/h) in glycerol-defined medium were 26.4% higher than the heparosan production (148 mg/L) and heparosan productivity (4.11 mg/L/h) in glucose-defined medium. DO-stat feeding approach as compared to pH-stat feeding, exponential feeding, exponential combined with pH-stat feeding, and constant rate feeding gave the highest heparosan titer at 8.63 g/L, which was nine times that of batch culture. The obtained optimal glycerol feeding strategy may be useful for the scaling-up of microbial heparosan production.

Keywords Heparosan · Feeding strategy · Glycerol · *Escherichia coli* K5

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Introduction

Heparin, clinically used as anticoagulation and antithrombotic agent, is one of the most important glycosaminoglycan drugs. Heparin possesses wide-range of potential applications in anti-infection, anti-inflammatory and anticancer drug development [1]. Heparin is currently extracted from porcine intestines or bovine lung [2] and there is a growing concern over the use of animal-derived components in biomedical and pharmaceutical applications. Moreover, traditional extraction process usually results in heavy environmental pollution. Chemical synthesis to produce heparin requires more than 60 steps with yield as low as 0.5% [3]. It is difficult to generate chemical heparin larger than a hexasaccharide if only by chemical synthesis [4]. Hence, bioengineered heparin from microbial heparosan has emerged as a new alternative for heparin production.

Heparosan is an acidic polysaccharide, comprised a (-GlcUA-1, 4-GlcNAc-1, 4-)$_n$ repeating disaccharide unit [5]. *Escherichia coli* K5 and *Pasteurella multicida* are able to produce heparosan as bacteria capsule [5, 6]. Kuberan et al. utilize heparan sulfate biosynthetic enzymes to synthesize heparan sulfate from *E. coli* K5 heparosan containing AT-binding sites. This demonstrates that the yield (~1.1%) of bioengineered heparin is higher by chemoenzymatic approach [7–9]. *E. coli* K5 heparosan is *N*-deacetylated using NaOH, neutralized with HCl, and *N*-sulfonated with $(CH_3)_3N{\cdot}SO_3$, followed by three additional enzymatic modifications, namely, C_5-epimerization/2-O-sulfonation, 6-O-sulfonation and 3-O-sulfonation with C_5 epimerase/2-O-sulfotransferase, 6-O-sulfotransferase, and 3-O-sulfotransferase, and finally converted to anticoagulant heparin [9].

The molecular size of heparosan (10–20 kDa) obtained from *E. coli* K5 is closer to heparin compared with that

from *P. multicida* (10–20 kDa) [10], which offers a promising precursor for production of a bioengineered heparin. The backbone structure of heparosan can be carefully modified using chemoenzymatic steps to generate a product which is identical to animal-derived heparin. Therefore, obtaining large quantities of heparosan is the first and necessary step to produce bioengineered heparin. However, there are few reports regarding the microbial production of heparosan in the current literature [11, 12]. A heparosan yield up to 15 g/L has been reported in a glucose-defined medium with oxygen enrichment [12]. Acetate is produced when *E. coli* is grown in oxygen-limiting or in excess glucose concentration conditions [13] which may inhibit cell growth and synthesis of end-products. The use of glycerol as the carbon source can reduce or prevent the inhibition caused by acetate formation [13]. In addition, glycerol is the main by-product of biodiesel industry and can thus be a low-cost feedstock to produce heparosan [14].

In this work, the influence of glucose and glycerol as carbon sources on the production of heparosan by *E. coli* K5 was investigated. Different glycerol feeding approaches including pH-stat feeding, exponential feeding, exponential combined with pH-stat feeding, constant rate feeding and DO-stat feeding were applied to improve the production and productivity of heparosan. The results obtained here may be useful for the industrial production of heparosan.

Materials and methods

Microorganism and media

E. coli K5 was frozen and stored in 1 mL glycerol or a glucose seed culture media containing 18% glycerol. 0.5 mL of defrosted *E. coli* was inoculated to 25 mL of the above-mentioned storing media in a 250 mL shake flask. Cell growth of seed cultivation was detected by a spectrophotometer (UVmini-1240 spectrophotometer, Shimadzu Co., Kyoto, Japan) at 600 nm (OD_{600}) after an appropriate dilution. After the lag phase (OD_{600} slowly increased from 0.3 to 3.0), exponential growth phase (OD_{600} rapidly increased from 3.0 to 17.0), and stationary phase (OD_{600} was stable around 17.0), the maximum OD_{600} reached 17.0. The culture broth was harvested for inoculation when cell growth was in middle exponential growth phase (OD_{600} reached 10.0) at about 12–16 h of cultivation.

Seed culture medium consisted of (g/L): glycerol 15 (carbon level component: 5.9 g/L) or glucose 15 (carbon level component: 6.0 g/L), $(NH_4)_2HPO_4$ 4, KH_2PO_4 13.5, citric acid 1.7, $MgSO_4 \cdot 7H_2O$ 1.4, thiamine-HCl 4.5 mg and 10.0 mL of trace metal solution. Trace metal solution consisted of (per liter of 5 M HCl): $FeSO_4 \cdot 7H_2O$ 10.0, $CaCl_2$ 2.0, $ZnSO_4 \cdot 7H_2O$ 2.2, $MnSO_4 \cdot 4H_2O$ 0.5, $CuSO_4 \cdot 5H_2O$ 1.0, $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ 0.1 and $Na_2B_4O_7 \cdot 10H_2O$ 0.02.

Glucose-defined fermentation medium contained (g/L): glucose 20 (carbon level component: 8.0 g/L), $(NH_4)_2HPO_4$ 4, KH_2PO_4 13.5, citric acid 1.7, $MgSO_4 \cdot 7H_2O$ 1.4, thiamine-HCl 4.5 mg and 10.0 mL of trace metal solution.

Glycerol-defined fermentation medium contained (g/L): glycerol 20 (carbon level component: 7.8 g/L), $(NH_4)_2HPO_4$ 4, KH_2PO_4 13.5, citric acid 1.7, $MgSO_4 \cdot 7H_2O$ 1.4, thiamine-HCl 4.5 mg and 10.0 mL of trace metal solution.

Feeding solution contained (g/L): glycerol 500 (carbon level component: 195.0 g/L), $MgSO_4 \cdot 7H_2O$ 20, and thiamine-HCl 0.2.

Heparosan production by *E. coli* K5 in shake flask

Heparosan production by *E. coli* K5 with glucose-defined medium and glycerol-defined medium was performed in 500 mL shake flasks. The *E. coli* grown on glycerol seed medium or glucose seed medium were inoculated into glycerol-defined medium and glucose-defined medium, respectively. Two milliliters of seed culture, which was pre-cultured in 250 mL shake flask that containing 25 mL glycerol-defined or glucose-defined seed medium, was added into 500 mL shake flask with 50 mL glycerol-defined or glucose-defined fermentation medium when the OD_{600} of seed culture reached 10. The culture was shaken at 200 rpm on a bacterial culture shaker at 37 °C for 36 h.

Effects of temperature and pH on cell growth and heparosan production were examined to obtain the optimum temperature and pH for batch and fed-batch culture of *E. coli* K5.

Batch and fed-batch culture of *E. coli* K5 in a 3-L fermentor

The production of heparosan by batch culture of *E. coli* K5 was performed with an initial glycerol concentration of 20 g/L. One hundred milliliters of seed culture that was pre-cultivated in 500 mL flasks was added into the 3-L fermentor (BioFlo 115, New Brunswick Scientific Co., Edison, NJ, USA) that containing 0.9 L glycerol-defined fermentation medium, when the OD_{600} of seed culture reached 10.0. Agitation was provided by two six bladed disk turbines. The pH was controlled automatically at 7.0 via the addition of 25% aqueous ammonia and the temperature was maintained at 37 °C. The aeration rate and agitation speed were 1.0 vvm and 400 rpm, respectively.

All fed-batch cultures were initiated as a batch culture with an initial glycerol concentration of 10 g/L, and the feeding solution was pumped into the fermentor using a

computer-coupled peristaltic pump. The dissolved oxygen was maintained approximately at 20% of air saturation by increasing the stirrer speed, and the aeration rate was 1.0 vvm.

In the pH-stat fed-batch culture, when glycerol was depleted and pH rose rapidly to 7.1, 20 mL of the feeding solution was added at a rate of 10 mL/min, and a total of 380 mL of feeding solution was added during the whole pH-stat fed-batch culture.

In the DO-stat fed-batch culture, when glycerol was depleted and DO increased sharply, 50 mL of feeding solution was added at a rate of 10 mL/min, and 500 mL of feeding solution was added during the DO-stat fed-batch culture.

In the exponential fed-batch culture, the substrate feeding rate was determined via Eq. 1, which was derived from a mass balance with the assumption of constant cell yield on the substrate and constant maintenance coefficient throughout fermentation [13]. F at a varied specific growth rate was given by

$$F = \mu V_0 X_0 \exp(\mu t)/Y_{X/S}(S_F - S) \quad (1)$$

where F is the glycerol feeding rate (mL/h), V_0 is the initial culture volume (L), μ is the specific growth rate (h^{-1}), X_0 is the initial cell concentration (g/L), $Y_{X/S}$ is the theoretical cell yield on glycerol (0.45 g/g), S_F is the glycerol concentration in the feeding solution (500 g/L), and t is the culture time. The end of glycerol consumption was detected by a sudden increase in both DO and pH values, and then the exponential fed-batch culture was started. Using experimental data from various fed-batch cultures of *E. coli* K5 in the glycerol unlimited conditions, the appropriate equation correcting μ with t was obtained as shown in Eq. 2:

$$\mu = -0.004(t - t_0)^2 - 0.03(t - t_0) + 0.47 \quad 0.14 < \mu < 0.47 \quad (2)$$

where μ is the specific growth rate (h^{-1}), t is culture time (h), and t_0 is the time of start feeding (h). The feeding process stopped at 20 h after feeding started as pre-set in the computer-coupled peristaltic pump, a total of 480 mL of feeding solution was added into the fermentor. The specific growth rate was pre-set as shown in Table 1.

Table 1 The specific growth rate set in exponential fed-batch culture

t_f/h	1	2	3	4	5	6	7–20
μ/h^{-1}	0.47	0.44	0.39	0.34	0.29	0.22	0.13

t_f feeding time, μ specific growth rate (h^{-1})

In the exponential combined with pH-stat fed-batch culture, the end of glycerol consumption was detected by a sudden increase in both DO and pH values, and then the exponential fed-batch culture was started. Exponential feeding was stopped whenever glycerol was added to the fermentor to 20 g/L and then pH change was observed. When pH rose above 7.1 due to the depletion of substrate, feeding was restarted [13].

In constant feeding rate fed-batch culture, the feeding solution was pumped into the fermentor at a feeding rate of 20 mL/h when glycerol was depleted and DO increased sharply, and a total of 300 mL of feeding solution was added into the fermentor.

Analytical methods

Ten milliliters of sample was taken from the fermentor, and 3 mL was used to determine OD with a spectrophotometer (UVmini-1240 spectrophotometer, Shimadzu Co., Kyoto, Japan) at 600 nm after an appropriate dilution. Five milliliters of sample was centrifuged at 8,000×*g* for 10 min to separate the supernatant from *E. coli* K5 cells, and the supernatant was used for the analysis of heparosan concentration. Dry cell weight (DCW) was proportional to OD_{600} and the correlated equation was

$$\text{DCW (g/L)} = 0.36 \times OD_{600}.$$

The samples taken from the shake flasks were centrifuged at 8,000×*g* for 10 min. One milliliter of supernatant was mixed with twofold of ethanol, followed by centrifugation at 8,000×*g* for 15 min. The precipitation was then dissolved in deionized water to an appropriate dilution for heparosan determination. Heparosan concentration was measured by the carbazole method based on uronic acid determination [15].

The glycerol concentration in the supernatant was determined by glycerol phosphate oxidase–peroxidase (GPO–POD) glycerol enzymatic rapid test kits purchased from Sigma Aldrich (China). Glucose concentration in the supernatant was measured by glucose-glutamate analyzer SBA-40C (Biology Institute of Shandong Academy of Sciences, Jinan, China).

Fermentation classification was characterized by fitting data from shake flask studies and each bioreactor feeding strategy to Leudeking–Piret equation. The growth, mixed or non-growth-associated characteristics were shown based on data fitting to Eq. 3.

$$q_p = \alpha\mu + \beta \quad (3)$$

where q_p is the specific heparosan production rate [mg/(g h)], α is the growth-associated coefficient (mg/g cell), μ is the specific growth rate (h^{-1}) and β is the non-growth-associated coefficient [mg/(g h)].

Results and discussion

Kinetics of cell growth and heparosan production in shake flask culture and 3-L fermentor

Figure 1 shows the kinetics of cell growth and heparosan production in glucose-defined medium and glycerol-defined medium. The maximum DCW in glycerol-defined medium (5.81 g/L) was 9.4% higher than that in glucose-defined medium (5.31 g/L). The maximum heparosan production in glycerol-defined medium (187 mg/L) was 26.4% higher than that in glucose-defined medium (148 mg/L). The average specific growth rate (0.04 h^{-1}) and average specific heparosan production rate (1.80 mg/g cell/h) in glycerol-defined medium were 33.3 and 56.5% higher than those (0.03 h^{-1} and 1.15 mg/g cell/h) in glucose-defined medium, respectively. Heparosan productivity in glycerol-defined medium (5.19 mg/L/h) was 26.4% higher than that in glucose-defined medium (4.11 mg/L/h). Carbon sources were both depleted at 12 h using glucose or glycerol as carbon source. The pH decreased from 7.0 to 6.0 at early stage both in glucose-defined medium and glycerol-defined medium. Sharp rises of pH were observed when carbon sources were both exhausted at 12 h. And the final pH values were as high as 8.5 and 8.2 in glucose-defined medium and glycerol-defined medium. Compared with pH variation in glucose-defined medium, pH in glycerol-defined medium varies within a small range, which may be more favorable for cell growth and heparosan production. These results demonstrated that glycerol was more favorable for cell growth and heparosan production compared to glucose. The influence of different initial glycerol concentrations on cell growth and heparosan production was further examined (Fig. 2a). The maximum heparosan production was obtained with a glycerol concentration of 20 g/L (carbon level component: 7.8 g/L).

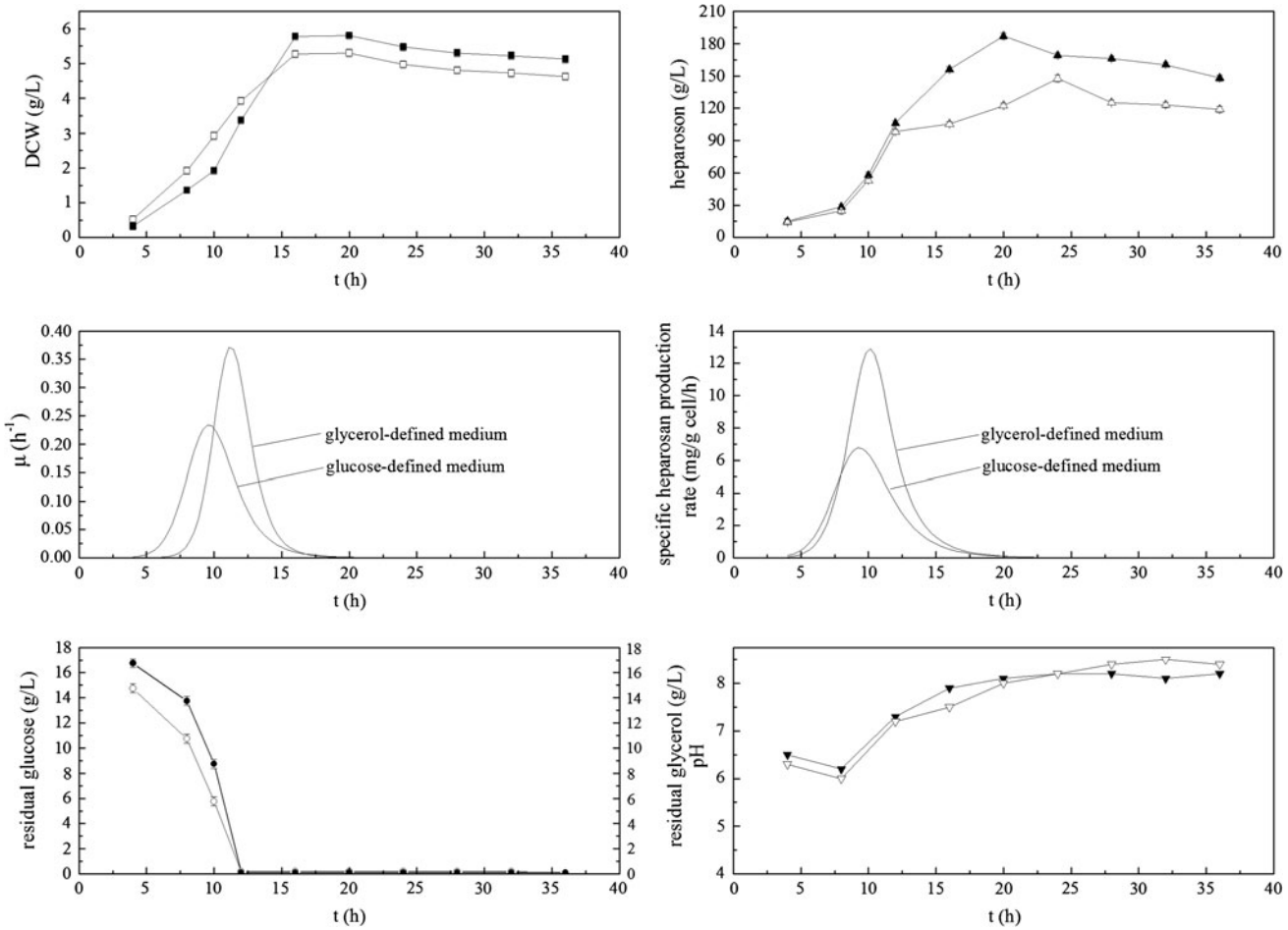

Fig. 1 Effects of glucose and glycerol on cell growth and heparosan production. *Filled squares* dry cell weight (DCW) in glycerol-defined medium, *blank squares* dry cell weight (DCW) in glucose-defined medium; *filled triangles* heparosan production in glycerol-defined medium, *blank triangles* heparosan production in glucose-defined medium; *filled circles* residual glycerol in glycerol-defined medium, *blank circles* residual glucose in glucose-defined medium; *filled inverted triangles* pH in glycerol-defined medium, *blank inverted triangles* pH in glucose-defined medium

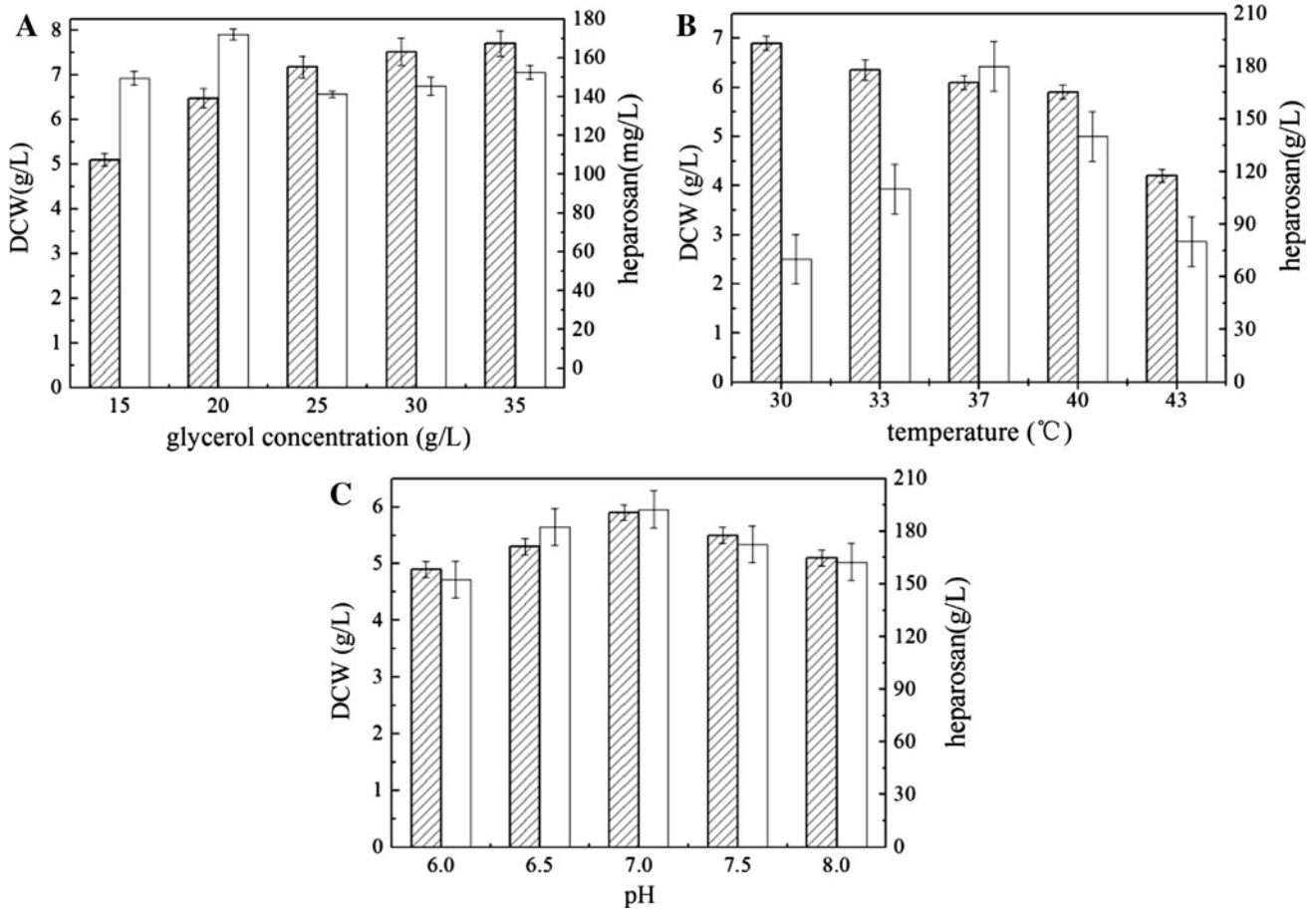

Fig. 2 Effects of glycerol concentration, temperature, and pH on cell growth and heparosan production. **a** Effects of glycerol concentration on cell growth and heparosan production *lined bars*: dry cell weight (DCW); *blank bars* heparosan. **b** Effects of temperature on cell growth and heparosan production; *lined bars* dry cell weight (DCW); *blank bars* heparosan. **c** Effects of pH on cell growth and heparosan production; *lined bars* dry cell weight (DCW); *blank bars* heparosan

Though DCW increased with the increase of glycerol concentration, increasing glycerol concentration in medium did not enhance heparosan production. Thus, feeding glycerol in fermentation process may be an efficient approach to obtain higher heparosan titer.

Effects of temperature and pH on cell growth and heparosan production were examined. As shown in Fig. 2b, the optimum temperature for heparosan production was 37 °C (180.0 mg/L). Although DCW of *E. coli* K5 grown at 30 °C (6.9 g/L) was 13.1% higher than that of *E. coli* K5 grown at 37 °C (6.1 g/L), heparosan production in 30 °C (70 mg/L) was 61.1% lower. As shown in Fig. 2c, the highest DCW and heparosan production at pH 7.0 were 5.9 g/L and 192.5 mg/L, respectively. Therefore, the temperature 37 °C and pH 7.0 were used in batch and fed-batch culture of *E. coli* K5 in a 3-L fermentor.

Figure 3 shows comparison of kinetics of cell growth and heparosan production in shake flask culture and 3-L fermentor. Maximum DCW in 3-L fermentor reached 7.2 g/L, which is nearly 23.9% higher than that in the shake flask. Heparosan was continuously synthesized from 4 h to 20 h in 3-L fermentor, maximum heparosan production was 740 mg/L, which is nearly four times that in the shake flask. Residual glycerol decreased to 4.0 g/L at 8 h, glycerol consumption rate was faster than that in the shake flask. The pH decreased from 7.0 to 6.0 at early stage of fermentation process in 3-L fermentor. Sharp rises of pH were observed when carbon source was exhausted after 8 h. The final pH reached 8.5. Both DCW and heparosan production decreased with the continuous increase of pH, which may be unfavorable for cell growth and heparosan production in later stage of fermentation. Therefore, pH was controlled at a constant value (pH 7.0) in further studies.

Effects of feeding strategies on cell growth of *E. coli* K5

Figure 4 shows the influence of different culture modes (batch cultivation, pH-stat fed-batch, exponential fed-batch, exponential combined with pH-stat fed-batch, constant

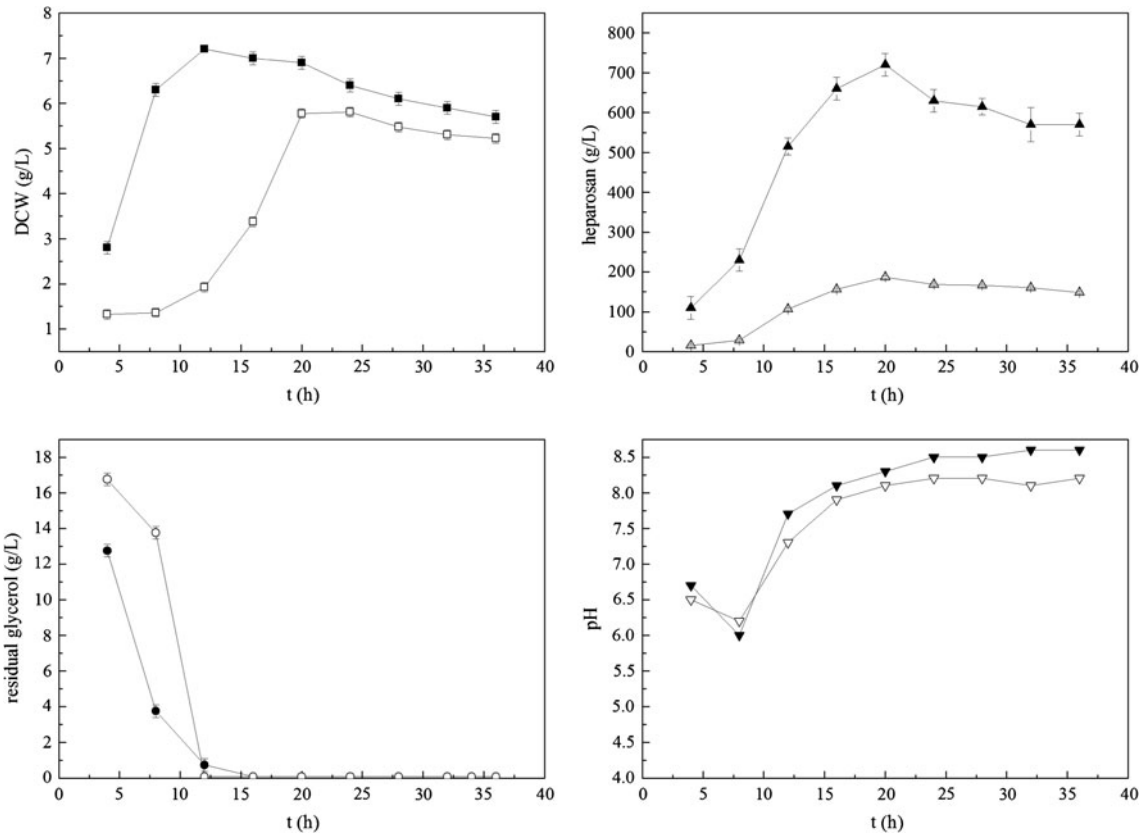

Fig. 3 Comparison between heparosan production in shake flask and 3-L fermentor. *Filled squares* dry cell weight (DCW) in 3-L fermentor, *filled triangles* heparosan production in 3-L fermentor, *filled circles* residual glycerol in 3-L fermentor, *filled inverted triangles* pH in 3-L fermentor; *blank squares* dry cell weight (DCW) in shake flask, *blank triangles* heparosan production in shake flask, *blank circles* residual glycerol in shake flask, *blank inverted triangles* pH in shake flask

feeding rate fed-batch and DO-stat fed-batch) on cell growth. In batch cultivation, the maximum DCW reached 8.63 g/L at 11 h, whereas in pH-stat fed-batch cultivation, the maximum DCW reached 45.83 g/L at 68 h. The pH-stat was a feedback control strategy that coupled nutrient feeding with the measurement of pH, based on the fact that pH increased when the carbon source was depleted. In a defined medium, the pH-stat did not respond rapidly when nutrient was depleted as it could be too tight on the culture, leading to a long lag time [13]. The repeated glycerol starvation in pH-stat cultivation resulted in longer culture time. In exponential fed-batch culture, the maximum DCW reached 67.58 g/L at 28 h, and resulted in the highest DCW in all feeding approaches conducted in this work. Average specific growth rate of exponential fed-batch culture was 0.14 h^{-1}, which is the highest in all feeding approaches. The maximum DCW and average specific growth rate of exponential fed-batch culture were nearly eight times and two times that in the batch culture, respectively, indicating that exponential fed-batch cultivation was efficient to obtain high cell concentrations. In exponential combined with pH-stat fed-batch culture, the maximum DCW reached 61 g/L at 32 h. The success of the exponential feeding is highly dependent on the parameters, including μ and $Y_{X/S}$. This system is, therefore, usually compensated by the feedback loop. Thus, exponential combined with pH-stat strategy was employed to ensure the success of exponential feeding [16]. The maximum DCW of exponential combined with pH-stat fed-batch culture (60.58 g/L) was nearly six times higher than that of the batch cultivation. In constant feeding rate fed-batch cultivation, the maximum DCW reached 47.85 g/L at 28 h. Average specific growth rate was 0.12 h^{-1} in exponential combined with pH-stat fed-batch. The maximum DCW of DO-stat fed-batch cultivation reached 65.01 g/L at 41 h, which was 7.5 times of that in the batch culture, and resulted in the highest DCW in all feeding approaches conducted in this work. Average specific growth rate was 0.12 h^{-1} in DO-stat feeding approach. Therefore, DO-stat feeding approach is an efficient strategy to maximize the cell density and heparosan titer.

Effects of feeding strategies on heparosan production by *E. coli* K5

Figure 5 shows the influence of different culture modes on heparosan production of *E. coli* K5. Data from shake flask

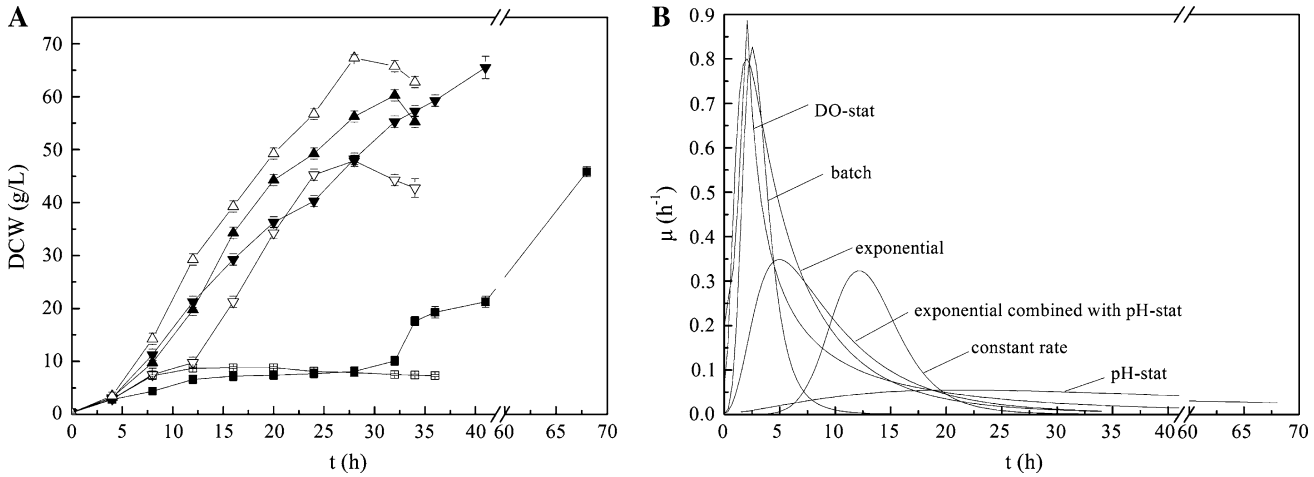

Fig. 4 Time courses of dry cell weight in different culture modes. *Blank squares* batch cultivation; *filled squares* pH-stat fed-batch cultivation; *blank triangles* exponential fed-batch cultivation; *filled triangles* exponential combined with pH-stat fed-batch cultivation; *blank inverted triangles* constant fed-batch cultivation; *filled inverted triangles* DO-stat fed-batch cultivation

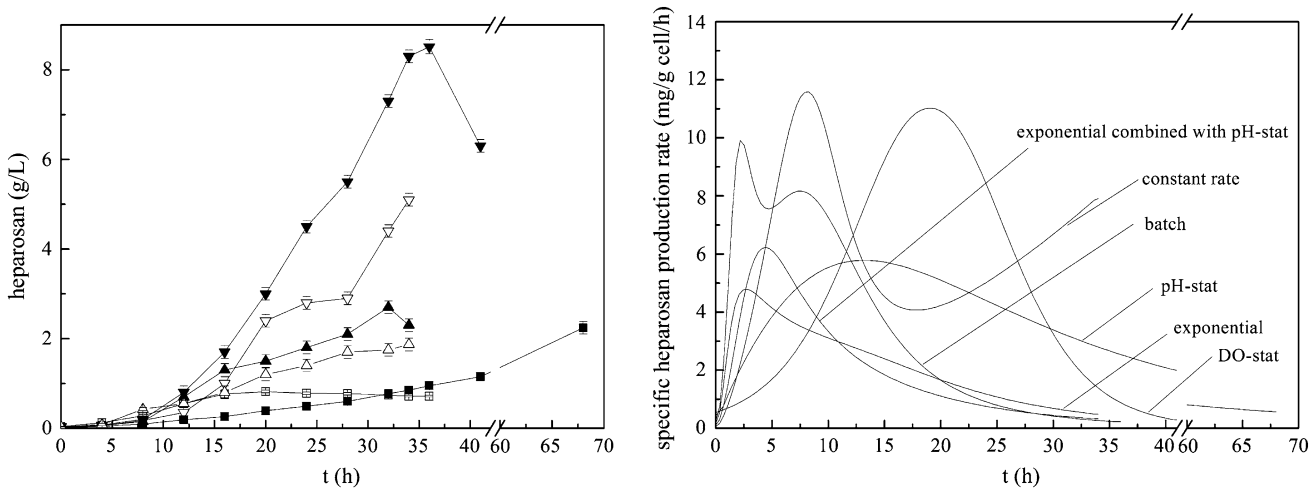

Fig. 5 Time courses of heparosan production in different culture modes. *Blank squares* batch cultivation; *filled squares* pH-stat fed-batch cultivation; *blank triangles* exponential fed-batch cultivation; *filled triangles* exponential combined with pH-stat fed-batch cultivation; *blank inverted triangles* constant fed-batch cultivation; *filled inverted triangles* DO-stat fed-batch cultivation

studies and each bioreactor feeding strategy were fitted to Leudeking–Piret equation to characterize relationship between cell growth and heparosan synthesis. The results were shown in Table 2. Heparosan synthesis is partially growth associated based on the data fitting to Leudeking–Piret equation (Eq. 3). Expression level of *SlyA*, which promotes the transcription level of K5 capsule gene cluster, increased rapidly during growth and reached maximal at the late exponential phase, while decreased during stationary phase. This may be the reason why the production rate of heparosan rapidly increased during exponential phase and decreased after exponential phase. Accordingly, the heparosan production is partially growth associated. Therefore, in order to increase heparosan production, not only enhancing cell density but also providing favorable heparosan synthesis environment should be considered.

Table 2 Parameters of Leudeking–Piret equation of shake flask studies and each feeding strategy

Culture mode	α (mg/g cell)	β [mg/(g h)]	R^2
Batch culture	10.55	2.67	0.84
pH-stat fed-batch	104.79	1.36	0.74
Exponential fed-batch	5.68	1.33	0.85
Exponential combined with pH-stat fed-batch	16.33	0.29	0.98
Constant feeding rate fed-batch	9.37	4.88	0.82
DO-stat fed-batch	17.46	4.66	0.88

In batch cultivation, heparosan accumulated gradually during exponential phase and stationary phase from 0 to 20 h and reached a maximal value of 0.82 g/L at 20 h.

The heparosan productivity, average specific heparosan production rate, and production per DCW were 22.78 mg/(L h), 3.42 mg/(g h) and 95.02 mg/g, respectively.

In pH-stat fed-batch cultivation, pH-stat did not respond rapidly when nutrient was depleted, resulting in a long lag time, and heparosan productivity [32.97 mg/(L h)] was only 44.73% higher than that in batch cultivation. In exponential fed-batch cultivation, the heparosan production per DCW (30.87 mg/g) and average specific heparosan production rate [2.14 mg/(g h)] were the lowest in all batch and fed-batch cultivations conducted in this work. Compensated by the pH feedback loop, exponential combined with pH-stat fed-batch culture obtained higher maximum heparosan production (2.67 g/L) and average specific heparosan production rate [2.32 mg/(g h)], which were 42.8 and 8.4% higher than that of exponential fed-batch cultivation, respectively. The results suggested that exponential fed-batch cultivation was not a suitable strategy to obtain high heparosan concentration. The reason why the heparosan production and the heparosan production per DCW were the lowest in all culture modes was that heparosan synthesis and cell growth shared precursors, such as glucose-1-phosphate, UDP-glucose and UDP-*N*-acetylglucosamine [17]. Because glycerol and some common components, which were precursors both in cell wall biosynthetic pathway and heparosan biosynthetic pathway, were preferentially used for cell growth, and less glycerol was used for heparosan synthesis. Therefore, the high cell density with low heparosan production was obtained by exponential feeding mode and exponential combined with pH-stat feeding mode. Accordingly, exponential fed-batch cultivation and exponential combined with pH-stat cultivation were favorable for cell growth, but not good strategies for heparosan production. In constant feeding rate fed-batch culture, the maximum heparosan production reached 5.09 g/L. And heparosan production per DCW was 106.33 mg/g, which was 1.2 times, 2.4 times, and 2.2 times that of pH-stat fed-batch cultivation, exponential fed-batch cultivation, and exponential combined with pH-stat fed-batch cultivation, respectively. The concentrated glycerol was continuously fed into the fermentor from 5 to 20 h, and sufficient glycerol was supplied for cell growth and heparosan production. Glycerol and some common components were precursors both in cell wall and heparosan biosynthetic pathways; when glycerol concentration was controlled at a low level, glycerol was preferentially used for cell growth, and less glycerol was used for heparosan synthesis. When sufficient glycerol was supplied, the competition of common precursors between heparosan synthesis and cell growth was avoided, thus more heparosan was synthesized during fermentation process. Therefore, the higher heparosan titer was obtained in constant feeding rate fed-batch cultivation. DO-stat fed-batch cultivation resulted in the highest heparosan production of 8.63 g/L and the highest average specific heparosan production rate of 5.99 mg/(g h). As shown in Figs. 5 and 6, glycerol concentration was maintained at 0–25 g/L, and the competition of common precursors between heparosan synthesis and cell growth was avoided in the presence of sufficient glycerols, and thus more heparosan was produced. Meanwhile, the DO-stat responded more rapidly to nutrient depletion than pH-stat in a defined medium [13], which can ensure feeding timely and avoid starvation of the cells. In this work, the glycerol was used as carbon source in fermentation medium, and the concentrated glycerol was fed into the fermentor using DO-stat feeding approach. Glycerol was fed into the fermentor timely when glycerol depleted. Therefore, there were enough nutrients for cell growth and heparosan production. Dry cell weight and heparosan production were both enhanced significantly by DO-stat feeding approach. Accordingly, DO-stat fed-batch cultivation was an efficient strategy for heparosan production by *E. coli* K5.

Effects of feeding strategies on glycerol consumption

Figure 5 shows the influence of different culture modes on glycerol consumption of *E. coli* K5. In batch culture, glycerol was consumed for cell growth, heparosan production and maintenance metabolism, and the final concentration of glycerol decreased from an initial concentration of 20 to 0.3 g/L. Cell yield on glycerol and heparosan yield on glycerol were 0.43 g/g and 41.00 mg/g, respectively. In pH-stat feeding mode, glycerol concentration was maintained between 0 and 12 g/L, as feeding solution was added as a pulse when pH increased rapidly during the fermentation process. In exponential fed-batch cultivation, glycerol concentration decreased from an initial concentration of 10–1.5 g/L during 0–5 h. Glycerol concentration was maintained below 2 g/L from 6 to 20 h. Glycerol accumulated from 20 to 34 h. In exponential combined with pH-stat fed-batch cultivation, glycerol concentration decreased from the initial 10 –2 g/L, and glycerol concentration was maintained below 2 g/L during the culture process. As shown in Fig. 5, glycerol concentration was successfully controlled at a low level to avoid the inhibition of cell growth in exponential combined with pH-stat strategy. In constant feeding rate fed-batch cultivation, glycerol concentration was accumulated to nearly 15 g/L at 20 h, and glycerol concentration decreased from 15 to 1 g/L at 34 h. Because glycerol feeding rate was higher than glycerol consumption rate, glycerol was thus accumulated in the broth from 5 to 20 h, and glycerol concentration decreased from 15 to 1 g/L after glycerol feeding stage stopped. In DO-stat feeding mode, glycerol concentration was maintained between 0 to 27 g/L, as

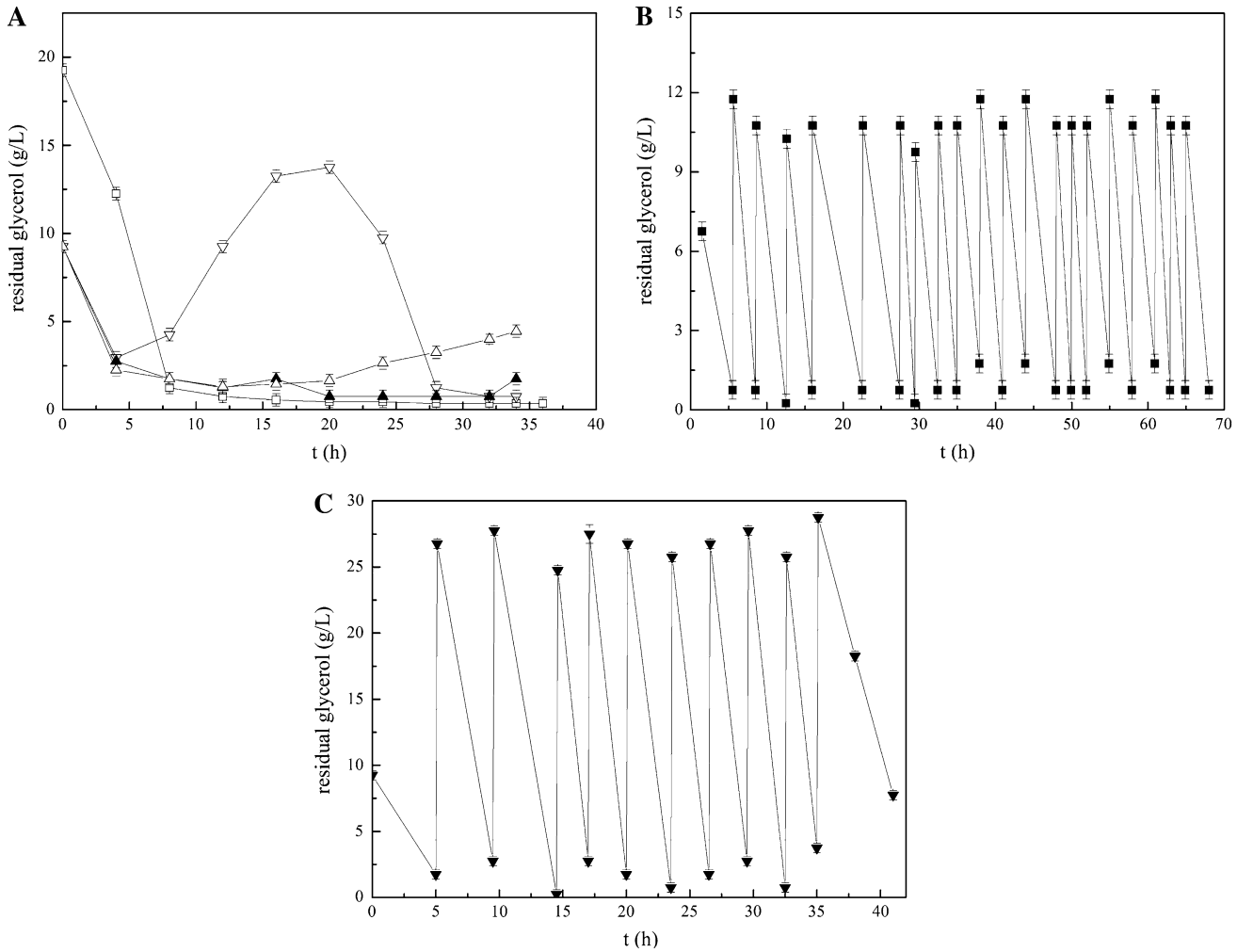

Fig. 6 Time courses of glycerol concentration in different culture modes. *Blank squares* batch cultivation; *filled squares* pH-stat fed-batch cultivation; *blank triangles* exponential fed-batch cultivation; *filled triangles* exponential combined with pH-stat fed-batch cultivation; *blank inverted triangles* constant fed-batch cultivation; *filled inverted triangles* DO-stat fed-batch cultivation

feeding solution was added as a pulse when pH increased rapidly during the fermentation process. Higher glycerol concentration is favorable for cell growth (as is shown in Fig. 2b). Therefore, maintaining glycerol concentration at a higher level was favorable for cell growth and heparosan production.

The comparison of recent work on heparosan production was shown in Table 3. In this work, the heparosan production of 8.63 g/L was achieved in DO-stat fed-batch cultivation, and the average specific growth rate and average specific heparosan production rate were 0.12 h^{-1} and 5.99 mg/(g h), respectively. And heparosan productivity [210.49 mg/(L h)] was higher than that recently reported [11]. Wang et al. [12] achieved a higher heparosan yield (15 g/L) in a fed-batch culture with glucose-defined medium, where pure oxygen was mixed with air to afford sufficient dissolved oxygen during the fed-batch cultivation. This may explain why a higher heparosan production can be achieved in comparison with this work, where no oxygen enrichments were used. However, it is difficult to use pure oxygen in industrial production and on the other hand, the achievement of higher heparosan production using pure oxygen indicates that dissolved oxygen is a key and limiting factor during heparosan production. Therefore, to further improve heparosan production, how to efficiently improve oxygen mass transfer is an important issue, and the use of oxygen vector or design of novel agitator with high mixing performance can be considered.

Conclusions

Effects of different glycerol feeding strategies including pH-stat feeding, exponential feeding, exponential combined with pH-stat feeding, constant rate feeding and DO-stat feeding on heparosan production by *E. coli* K5 was

Table 3 Comparison of different culture modes on heparosan production

Items	Batch culture	pH-stat fed-batch	Exponential fed-batch	Exponential combined with pH-stat fed-batch	Constant feeding rate fed-batch	DO-stat fed-batch
A. Kinetics of cell growth						
Maximum dry cell weight (g/L)	8.63	45.83	67.58	60.58	47.85	65.01
Maximum specific growth rate (h^{-1})	0.82	0.05	0.71	0.62	0.32	0.89
Average specific growth rate (h^{-1})	0.07	0.04	0.14	0.12	0.08	0.12
Cell productivity [g/(L h)]	0.21	0.67	1.78	1.78	1.41	1.59
B. Glycerol utilization						
Cell yield on glycerol (g/g)	0.43	0.23	0.28	0.30	0.30	0.25
Heparosan yield on glycerol (mg/g)	41.00	11.18	8.90	13.35	31.80	33.19
C. Heparosan production						
Maximum heparosan production (g/L)	0.82	2.24	1.87	2.67	5.09	8.63
Maximum specific heparosan production rate [mg/(g h)]	8.16	5.78	4.78	6.23	11.57	11.03
Average specific heparosan production rate [mg/(g h)]	3.42	2.81	2.14	2.32	5.97	5.99
Heparosan productivity [mg/(L h)]	22.78	32.97	54.94	78.53	149.65	210.49
Heparosan production per dry cell weight (mg/g)	95.02	48.77	30.87	44.07	106.33	132.75

studied in this work. The highest heparosan production (8.63 g/L), heparosan productivity [210.49 mg/(L h)] and heparosan production per DCW (132.75 mg/g) were obtained in DO-stat fed-batch cultivation. The obtained optimal feeding approach may be useful for the further scale-up of heparosan production and preparation of heparin in large quantities.

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