RESEARCH ARTICLE

Influence of medium constituents on the biosynthesis of cephalosporin-C

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Abbreviations: CPC: cephalosporin-C
                       STBR: stirred tank bioreactor

***Acremonium chrysogenum* NCIM 1069 was used for the biosynthesis of cephalosporin-C (CPC) in batch mode of cultivation. The effect of different medium constituents for better yield of CPC was thoroughly investigated. From the results of the fermentation, it was found that ammonium sulphate as inorganic nitrogen source and methionine at the concentration of 0.4% are most suitable for higher yield of antibiotic. The variation in the C/N ratio on the biosynthesis of CPC showed that a C/N ratio of 8.0 is most suitable for maximum production of CPC.**

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Cephalosporins together with penicillin belong to the family of β-lactam antibiotics. Cephalosporins are important broad-spectrum antibiotics in the international market and are more resistant to β-lactamases than penicillins. Cephalosporin-C (CPC), the starting material used for the synthesis of various cephalosporins is produced by aerobic fermentation using different strains of *Cephalosporium acremonium* and by streptomyces species like *Streptomyces clavuligerus*. The CPC has a weak antibacterial activity, but the modification of its side chain generates semisynthetic cephalosporins having diversified

antibacterial activity. The mold is recently renamed as *Acremonium chrysogenum* (Brakhage, 1998; Demain and Zhang, 1998; Silva et al. 1998; Schmitt et al. 2004; Tollnick et al. 2004). The beta-lactam antibiotics particularly penicillin and cephalosporins share approximately 65% of worldwide market of antibiotics (Elander, 2003). A number of reports are available on the various aspects of CPC production such as production in batch as well in continuous mode using free and immobilized cells of mold, and modes of bioreactor operation etc. (Srivastava and Kundu, 1999; Cruz et al. 2001; Ellaiah et al. 2002; Kundu et al. 2003; Cruz et al. 2004; Kim et al. 2005). The optimization of media components for CPC biosynthesis has been reported by few authors (Shen et al. 1984; Demain and Vaishnav, 2006) and different hypothesis has been proposed. Some of them have investigated the effect of a single constituent on the fermentation of CPC. We have investigated the influence of medium constituents (such as inorganic nitrogen sources, methionine etc.) on the production of CPC in detail. We have also optimized the carbon nitrogen ratio on the biosynthesis of CPC using *Acremonium chrysogenum*. Such kinds of studies are, nonetheless, very important to secure good production performance.

The biosynthesis of CPC is studied in a 2L-stirred tank bioreactor (STBR) using synthetic medium at optimal conditions. It was observed that with the progress of fermentation increase in the viscosity of broth occurs and thereby decreasing oxygen mass transfer. In our earlier communication, we have attempted to increase the oxygen transfer rate to the broth by coimmobilizing mold with algae called *Chlorella pyrenoidosa* in a symbiotic mode (Kundu et al. 2003). We have also studied the morphological differentiation and effect of seed of different age on the production of CPC (Verma et al. 2006).

The present paper deals with the optimization of different medium constituents for the increase in biosynthesis of CPC. A comparison of the findings of shake flask and bioreactor studies has also been made for better understanding of the CPC production process.

MATERIALS AND METHODS

Organism

The production of CPC was carried out by *A. chrysogenum* NCIM 1069, obtained from National Chemical Laboratory, Pune, India. The culture was maintained on potato-dextrose-agar (PDA) after incubation for 7 days at pH 7.2 and temperature 28ºC.

Culture medium and conditions

The culture was propagated in the seed medium containing (gL^{-1}) sucrose, 30; potassium dihydrogen orthophosphate, 1.5; magnesium sulphate, 0.3; and trace metal solution 15 mL which was sterilized separately (Srivastava et al. 1996).

The trace metal solution contained (gL^{-1}) ferrous ammonium sulphate, 0.15; magnesium sulphate, 0.367; calcium chloride, 0.075; zinc sulphate, 0.03; manganese sulphate, 0.03; and copper sulphate 0.008. The culture was grown in the seed medium for 6-7 days at 28ºC in an orbital shaker set at 200 rpm.

Batch fermentations were carried out in shake flask and in a STBR at 28ºC for 144 hrs on the synthetic production medium. The production medium contained (gL^{-1}) sucrose, 30; ammonium sulphate, 7.5; dipotassium hydrogen orthophosphate, 15.6; potassium dihydrogen orthophosphate, 15.3; DL-methionine, 3.0; sodium sulphate, 1.7 and oleic acid, 1.5. The pH of the medium was adjusted to 7.2 before sterilization.

The effects of various media constituents at different concentrations were studied and the production of CPC was monitored at shake flask level.

Five different inorganic nitrogen sources (ammonium sulphate, ammonium nitrate, potassium nitrate, sodium nitrate, and ammonium chloride respectively) were selected and their concentration were varied (keeping the total nitrogen concentration almost constant) to observe the effect on the yield of CPC.

The effect of methionine on the production of CPC was investigated by varying its concentration in the synthetic production medium in the range of 3.0 g/L to 5.0 g/L, keeping all other parameters constant.

In order to investigate the effect of C/N ratio on the biosynthesis of CPC, the concentration of sucrose was varied keeping the concentration of ammonium sulphate (as nitrogen source) constant. All other fermentation parameters of shake flask studies were kept constant (rpm 200, temperature 28ºC and time of fermentation 144 hrs).

Inoculum

The surface growth from a 7-days old slant was suspended in 5 mL of sterile distilled water and was used to inoculate the seed medium.

Cultivation in STBR

After optimization of various media constituents, batch studies of CPC production was performed at shake flask and bioreactor level (2.0 L capacity, Infors A.G. Switzerland, working volume 1.2 L) at optimal conditions. The samples were aseptically withdrawn at certain time intervals and analyzed for sucrose, CPC and dry cell weight. Sterile air was passed to the bioreactor for aeration and the temperature inside the vessel was maintained at 28ºC by cooling water passing through the jacket. The pH of the broth was maintained at 7.2 using 2M KOH and 2N HCl. The DO was maintained above 30% saturation by aeration and agitation.

Analysis

The residual sucrose was estimated after hydrolysis by DNS method (Miller, 1959). The method of Boxer and Everett (Boxer and Everett, 1949) was used for the estimation of CPC. Bioassay technique was also conducted for estimation of CPC using *Alcaligenes faecalis* NCIM 2105 as a test organism. The dry cell weight was estimated by centrifuging 10 mL of fermentation broth, washed twice with distilled water, recentrifuged and kept for drying at 80ºC till the constant weight.

RESULTS

Effect of different inorganic nitrogen sources on the biosynthesis of CPC

Figure 1, Figure 2, Figure 3, Figure 4 and Figure 5 show the findings of the effect of different inorganic nitrogen sources on the biosynthesis of CPC. It has been observed that the ammonium sulphate, when used as nitrogen source increases the production of CPC in comparison to other inorganic nitrogen sources. The increase in dry cell weight was also noticed. Potassium nitrate also gave good yield of CPC followed by ammonium nitrate. The minimum concentration of CPC was found with ammonium chloride as nitrogen source.

Effect of DL methionine on the biosynthesis of CPC

The results of the effect of different concentrations of methionine are presented in Figure 6. It is observed that the production of CPC increases with increase in the methionine concentration up to 4.0 g/L and thereafter started decreasing. It has been reported that methionine at certain level (optimum concentration) stimulates the differentiation of mold and increases the yield of CPC. Hence, in subsequent studies the optimum concentration of methionine used is 0.4%.

Effect of C/N ratio on the biosynthesis of CPC

The results of this experiment are given in Figure 7. It is clear from the figure that production of CPC increase from C/N ratio 5.33 to 8.0 and then decreased. The decreased yield of CPC at higher C/N ratio is due to accumulation of sucrose in the broth, which may likely inhibiting the synthesis of enzymes responsible for the formation of CPC.

Batch fermentation of CPC

It has been observed that the growth rate of mold started increasing after two days of fermentation and the maximum rate of cell mass was formed at around 72-96 hrs, after that the rate of cell mass formation decreased. The formation of CPC initially is low but the production increases steadily at the end of late exponential phase and reached to the maximum value at the stationary phase (Table 1). This evidently reflects that the production of CPC occurs at the late hrs of fermentation, *i.e.* idiophase. The production of CPC at bioreactor level was higher (0.69 mg/mL) as compared to shake flask (0.53 mg/mL). This increased production of CPC in bioreactor is because of aeration, better mixing and control of pH and dissolved oxygen (D.O.). A comparative evaluation of different biochemical process parameters of CPC production in shake flask and bioreactor is represented in Table 2.

DISCUSSION

Antibiotics are the secondary metabolites produced by the microbes. The biosynthesis of antibiotics *i.e.* the secondary metabolite is not associated with the growth of mold. In microbial cultures, the production of secondary metabolites and growth related functions do not occur simultaneously. It has been observed that during the production of secondary metabolites, the cell biomass of microbes increases initially and after certain time, it becomes constant/decreases slightly. An increase in the viscosity of the medium is also observed decreasing the oxygen transfer rate. Production varies with the constituents of the media and the stage of the culture development. The secondary metabolites accumulate only after the growth phase (tropophase) *i.e.* when the culture attains a specific growth rate. Secondary metabolites are often called "idiolites" since they are produced in the "idiophase".

This investigation on the biosynthesis of CPC was attempted with an aim to increase its production as it is a very important beta lactam antibiotic with a broad-spectrum activity. A thorough literature review was performed and it was noticed that though a number of highly productive strains and a number of different strategies have been used for better yield, not much attention has been given in the constituents of synthetic medium used for its biosynthesis. In addition, a number of other reports are available on the production of CPC using complex medium.

When different inorganic nitrogen sources were added to the synthetic production medium, it has been found that only a few are used by mold for maximum synthesis of CPC. Others are not utilized by mold at the same extent. Ammonium sulphate was observed to be the best source of inorganic nitrogen for the higher production of CPC. High content of nitrogen is found to decrease the production. It might be due to the reason that it interferes to the process of differentiation of mycelium to swollen hyphal fragments and arthrospores during the production stage. Production of CPC by many strains of *C. acremonium* is known to be stimulated by the addition of the amino acid methionine. Although methionine can supply sulphur to CPC biosynthetic pathway, this is not the sole mechanism of its stimulatory effect, since norleucine, a non-sulphur analogue also has similar effect. Two other compounds such as DL-methionine-DL-sulfoxide and S-methyl-L-cysteine were reported to be active towards CPC production (Demain and Newkirk, 1962). The methionine stimulates the synthesis of CPC by playing a role in differentiation process. Queener

and Ellis (Queener and Ellis, 1975) observed the effect of methionine concentrations during the formation of unicellular arthrospores while attempting the production of CPC in complex medium. They have observed that at higher concentration, the percentage of unicellular arthrospores increased from 55% to 80% and CPC production is decreased to 85% of that obtained with normal DL-methionine level. Similarly the role of methionine in CPC synthesis by *Acremonium chrysogenum* is investigated where the authors have proved that methionine plays a direct role by supplying sulphur for the formation of cephalosporins. Enzymatic and genetic studies of methionine have revealed that it induces four of the enzymes of CPC biosynthesis at the level of transcription. It is also converted to cysteine, one of three precursors of CPC, by cystathionine-gamma-lyase. The main effect of methionine on cephalosporin production results from its regulatory role, which can be duplicated by the non-sulphur analog norleucine. Eliminating cystathionine-gamma-lyase prevents the enhancing precursor effect of methionine on CPC production, and cystathionine-gamma-lyase overproduction in moderate doses increases CPC formation (Martin and Demain, 2002). When *A. chrysogenum* NCIM 1069 was used for the production of CPC, the yield of CPC decreased above optimum concentration of methionine (0.4%). This decrease in production of antibiotic above certain concentration might be due to its toxic effect on the mold. Production of secondary metabolites is often subjected to the regulation by the readily metabolizable carbon sources and a decrease in the titre is noted. Even at higher concentration of carbon source with a slow uptake rate, which is beneficial for the production (sucrose and high C/N ratio), the productivity of CPC decreased. This is probably because of accumulation of sucrose in the fermentation broth leading to the fragmentation and autolysis of the mold.

CONCLUDING REMARKS

The above studies conclude that for better yield of CPC, the nutritional requirement of mold should be at their optimal concentrations. It was found that ammonium sulphate as inorganic nitrogen source and methionine at the concentration of 0.4% are most suitable for higher yield of antibiotic. The variation in the C/N ratio on the biosynthesis of CPC showed that a C/N ratio of 8.0 is most suitable for maximum production of CPC. At higher concentrations (above optimal) the CPC biosynthesis decreased drastically and the cost economy will be increased.

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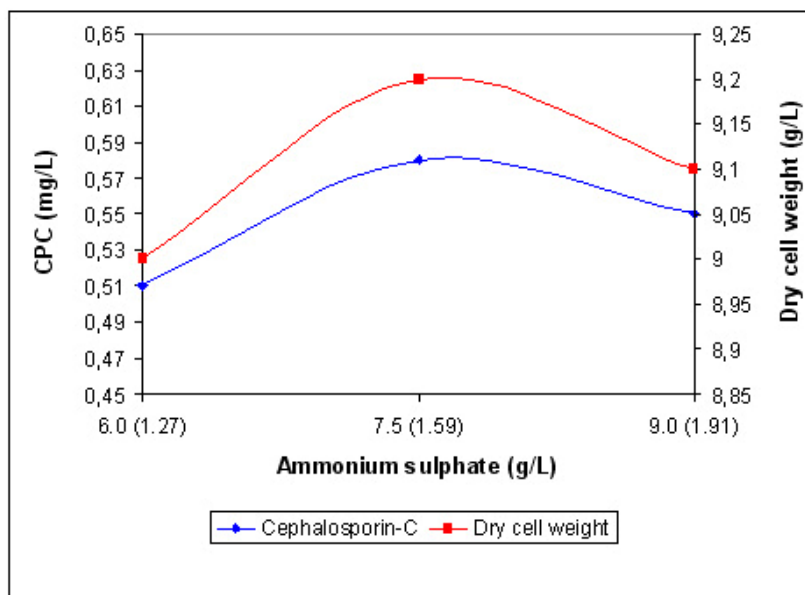
APPENDIX
FIGURES

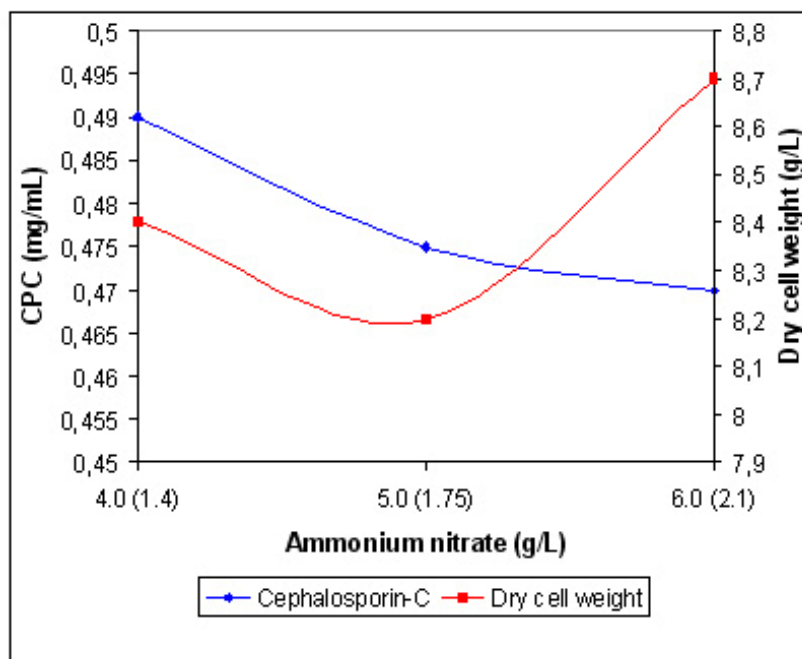**Figure 1. Effect of ammonium sulphate on the biosynthesis of CPC.**

Figure 2. Effect of ammonium nitrate on the biosynthesis of CPC.
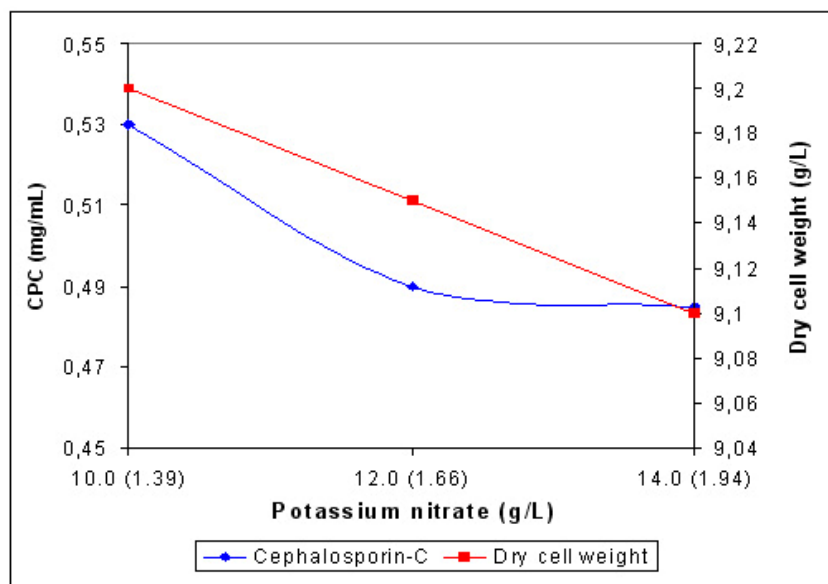
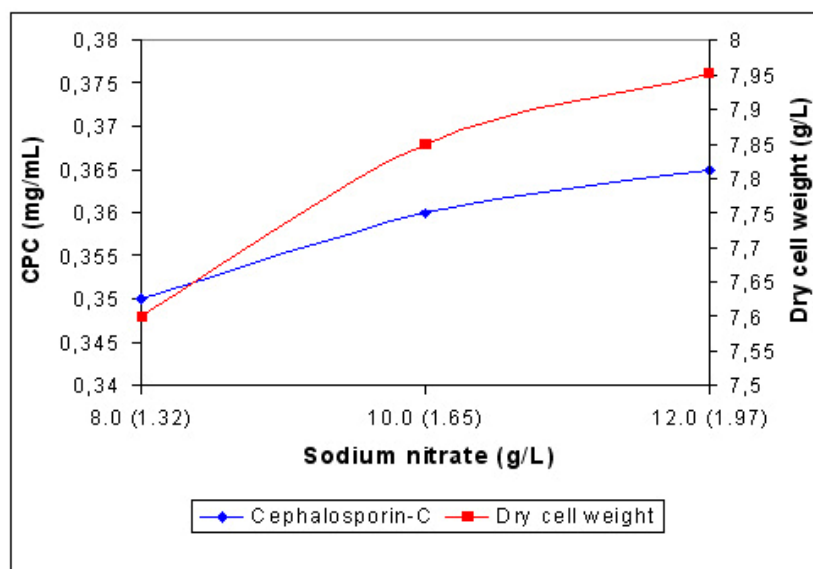**Figure 3. Effect of potassium nitrate on the biosynthesis of CPC.**

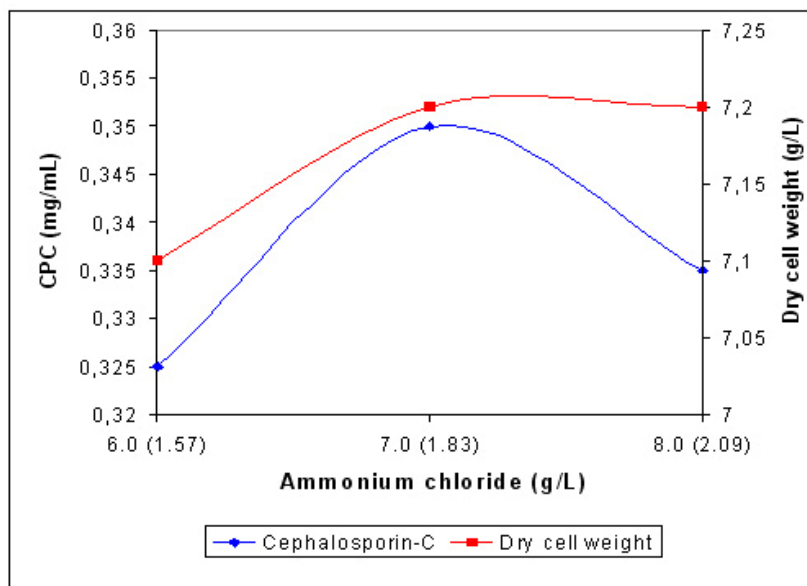**Figure 4. Effect of sodium nitrate on the biosynthesis of CPC.**

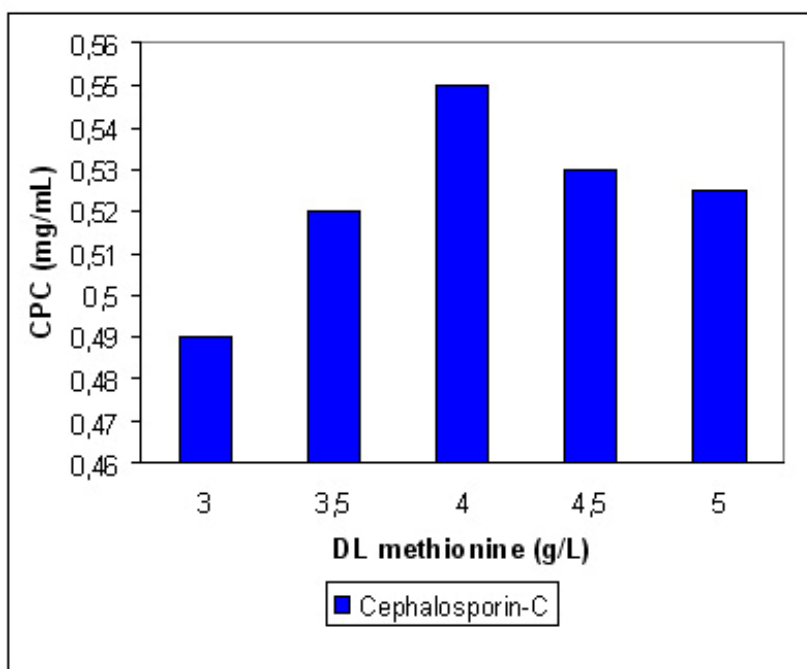**Figure 5. Effect of ammonium chloride on the biosynthesis of CPC.**

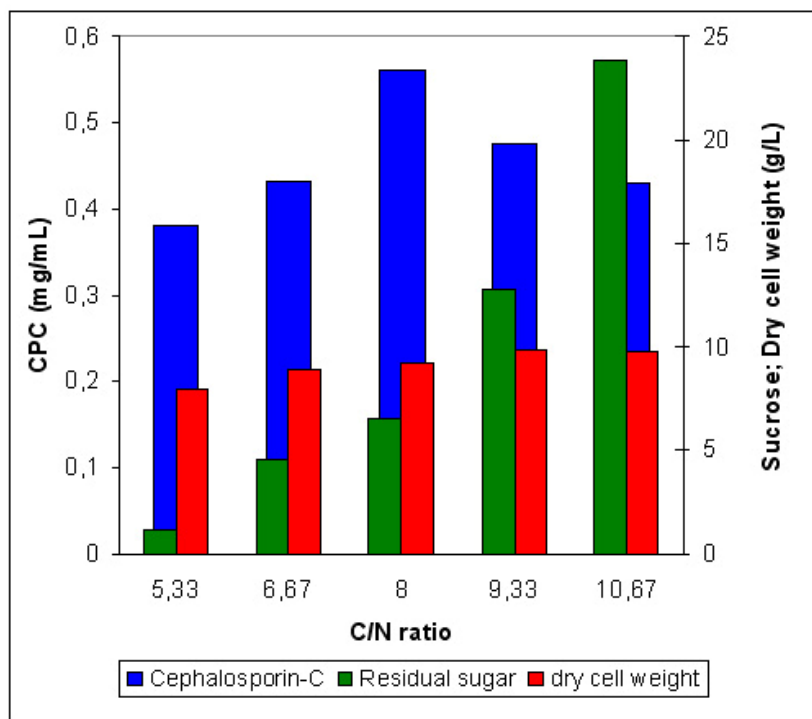**Figure 6. Effect of DL methionine on the biosynthesis of CPC.**

Figure 7. Effect of C/N ratio on the biosynthesis of CPC.

TABLES

Table 1. Batch fermentation of CPC in shake flask and in bioreactor.

Time (Days)	Residual sugar (g/L)		Dry cell weight (g/L)		CPC (mg/mL)	
	Shake flask	Bioreactor	Shake flask	Bioreactor	Shake flask	Bioreactor
0	31.5	32.0	1.08	1.24	0.00	0.00
1	27.2	26.5	2.65	2.80	0.025	0.055
2	22.4	20.0	4.96	5.26	0.148	0.22
3	17.5	17.2	6.24	7.80	0.225	0.38
4	14.0	13.4	7.14	8.82	0.322	0.482
5	10.2	8.5	8.50	9.88	0.450	0.620
6	6.8	4.6	8.60	10.6	0.518	0.696
7	4.9	2.7	8.32	10.4	0.532	0.640

Table 2. A comparative evaluation of shake flask and bioreactor performance.

Parameters	Shake flask	Bioreactor
Working volume	50 mL	1.2 L
Maximum cell biomass (Xm, g/L)	8.60	10.6
CPC (mg/mL)	0.53	0.69
Growth yield (Yx/s)	0.282	0.32
Productivity (Pr, g/L/h)	3.2×10^{-3}	4.8×10^{-3}