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The Effects of High Initial D-glucose and Ammonium Sulfate Concentrations on the Production of Cephalosporin C by *Cephalosporium acremonium* (ATCC 14615)

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The Effects of High Initial D-glucose and Ammonium Sulfate Concentrations on the Production of Cephalosporin C by Cephalosporium acremonium (ATCC 14615)

Summary : *The production of cephalosporin C (CPC) was achieved at high initial D-glucose and ammonium sulfate concentrations in growth medium by means of whole free cells of Cephalosporium acremonium (ATCC 14615) in cotton plugged shaker flasks. D-glucose and ammonium sulfate levels in fermentation broth were adjusted to 10-150 gL^{-1} and 7.5-100 gL^{-1}, respectively, and CPC, microorganism, dissolved oxygen, D-glucose concentrations and pH were determined in the liquid samples. Maximum antibiotic concentration and specific product yield constantly increased with increasing glucose concentration although maximum microorganism yield and specific growth rate started to decrease after experiencing a maximum due to the inhibition effect of higher glucose concentrations. Nevertheless, specific growth rate started to increase again denoting the capability of microorganisms to alter cell metabolism against substrate inhibition which was impossible to explain by any inhibition model existing in the literature. Maximum product yield and specific production rate showed identical oscillatory trends against inhibiting glucose levels. With increasing ammonium sulfate concentration, specific growth rate, maximum CPC concentration and specific product yield showed similar trends as those of glucose case while specific production rate and antibiotic yield did not present an oscillatory behavior.*

Key words: *Cephalosporin-C, Cephalosporium acremonium, ammonium sulfate, D-glucose, fermentation, inhibition.*

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Yüksek Başlangıç D-glikoz ve Amonyum Sülfat Derişimlerinin Cephalosporium acremonium (ATCC 14615) tarafından üretilen Sefalosporin C üzerine etkileri

Özet : *Yüksek D-glikoz ve amonyum sülfat besiyeri başlangıç derişimlerinde sefalosporin C (CPC) nin Cephalosporium acremonium (ATCC 14615) tarafından pamuk tıpalı erlenler içinde üretimi gerçekleştirilmiştir. Fermentasyon ortamında, D-glikoz ve amonyum sülfat derişimleri sırasıyla 10-150 gL^{-1} and 7.5-100 gL^{-1} aralığında değiştirilmiş ve sıvı örneklerde CPC, mikroorganizma, çözünmüş oksijen ve D-glikoz derişimleri ve pH saptanmıştır. Artan glikoz derişimiyle maksimum antibiyotik derişimi ve özgül ürün verimi düzenli olarak artarken, maksimum mikroorganizma verimi ve özgül büyüme hızı maksimum değerlerinden sonra yüksek glikoz derişimlerinin inhibisyon etkisi nedeniyle azalmıştır. Buna karşın özgül üretim hızı, mikroorganizmaların substrat inhibisyonuna karşı hücre metabolizmalarını değiştirmeleriyle literatürde var olan inhibisyon modelleriyle açıklanamaz bir şekilde tekrar artmıştır. İnhibisyon yaratan glikoz derişimlerinde maksimum ürün verimi ve özgül üretim hızı benzer salınımlarla değişmiştir. Artan amonyum sülfat derişimlerinde özgül üreme hızı, maksimum CPC derişimi ve özgül ürün verimi glikoz inhibisyonundakine benzer eğilimler gösterirken, özgül üretim hızı ve antibiyotik verimi öncekinden farklı olarak salınımlı değerler vermemiştir.*

Anahtar kelimeler: *Sefalosporin C, Cephalosporium acremonium, amonyum sülfat, D-glikoz, fermentasyon, inhibisyon.*

INTRODUCTION

The production of antibiotic activity by a particular strain of Cephalosporium was first noted by Brotzu[1]. Several antibiotics with different properties were isolated by Abraham and Newton and named cephalosporin C, N and P which were largely used in pharmaceutical industry[2,3]. Due to excellent an-

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tibacterial activity and broad spectrum of its semisynthetic derivatives, cephalosporin C is one of the most important antibiotics in pharmaceutical industry. For the production, fermentation is still the only means because synthetic production process is complex and less efficient [4].

Cephalosporin C resembles penicillin in possessing a fused ß-lactam ring with remarkably less toxicity and broader spectrum against Streptococci and certain gram-negative bacilli. Profiting from the experience with semisynthetic penicillins, scientists have used the 7-aminocephalo-sporanic acid, 7-ACA, prepared by enzymatic degradation of cephalosporin C to prepare thousands of semisynthetic cephalosporins [5,6].

Currently, cephalosporins are being produced by microbiological processes where special strains of Cephalosporium are selected which produce more cephalosporin C and less cephalosporin N than the parent culture. The growth of these in special complex fermentation media with modifications in processing has resulted in remarkable antibiotic titers[7].

In the present study, Cephalosporium acremonium (ATCC 14615) was selected as the most adequate strain for a chemically defined medium containing glucose, ammonium sulfate, yeast extract, peptone and various salts. It grows rapidly in shaking flasks without any agglomeration and thus provides great easiness in substrate and product mass transfer. Using this culture, the effects of high initial carbon and nitrogen substrate concentrations on specific growth and production rates, maximum product concentration and yield factors were investigated.

MATERIALS and METHODS

Microorganism

The strain Cephalosporium acremonium is a pink colored microorganism, 40-60 µm in length and reproduces by spore formation. Highly differentiating and homogeneously growing culture of *Cephalosporium acremonium* (ATCC 14615) was kindly donated by Glaxo Pharmaceutical Co. of UK and propagated on solid agar medium[8].

Culture medium and Cultivation Conditions

For fermentation and inoculation preparation, a broth was prepared with distilled de-ionized water of the following composition in g L^{-1}:

D-glucose (anhydrous), 30; ammonium sulfate, 7.5; Difco® yeast extract, 5.0; Difco® peptone, 0.5; KH_2PO_4, 3.0; K_2HPO_4, 4.5 and $MgSO_4.7H_2O$, 0.5. In the experiments for antibiotic fermentation, either D-glucose or ammonium sulfate concentrations were varied in the medium composition keeping the others unchanged. Medium pH was adjusted to 6.5 with dilute H_2SO_4 and the broth was sterilized at 121°C for 15 minutes prior to inoculation. All fermentations were carried out simultaneously in sponge-plugged 250 ml erlenmeyers placed in constant 27°C water bath shaker with 60 strokes/min shaking rate. The most appropriate inoculation percent has been determined as 10% (v/v) of 150 ml working volume for each flask. The inoculant was always in exponential growth phase when transferred and the standardization was achieved by determining the microorganism concentration and substrate consumption rate together. The fermentation was determined to be non-growth associated and always extended to the point where maximum antibiotic titer was achieved.

Method of Analysis

At appropriate time intervals, 10 ml of samples were taken aseptically from each erlenmeyer and centrifuged at 6000 rpm for 15 minutes for the determination of D-glucose, antibiotic and microorganism concentrations. Meanwhile, pH and dissolved oxygen concentrations were checked in every sample for appropriate values. pH was measured by a Pye-Unicam® Model 25 pH meter in the supernatant separated from the microorganism after centrifugation.

Dissolved oxygen concentration was determined with Gallencamp® DO meter in liquid samples. Microorganism concentration was determined by measuring the absorbance of the centrifuged microorganism after washing twice with distilled water at 450 nm using Spectronic 20-D Bausch & Lomb®

spectrophotometer. A calibration curve for optical density vs. dry weight was established a priori with a time course study of microorganism concentration vs. absorbance. Specific growth rate was calculated by having the ratio of time-derivative value of microorganism concentration to corresponding microorganism concentration for obtained data. Specific production rate was estumated by using the derivative values of Cephalosporin C concentration. D-glucose and cephalosporin C analysis were accomplished through YSI® Model 27 glucose analyzer and CECIL® Model 1100 series HPLC, respectively.

RESULTS and DISCUSSION

Fermentations were carried out simultaneously in triplicates and the corresponding results did not deviate from each other more than 6%, thus average results were presented in all the figures. In each experiment, initial pH changed insignificantly until maximum antibiotic concentration was realized. Dissolved oxygen concentrations were always higher than 1 ppm, reported to be the critical value. In all runs, the culture *Cephalosporium acremonium* (ATCC 14615) grew homogeneously without any lump formation. During the fermentations, maximum cell concentrations were realized between 1.53 - 3.00 gL^{-1} in fermentation periods of 120-170 hours. Specific growth rate, μ, was calculated by least square regression of the linear data in the graph ln (X) vs. t. Specific production rate, ν, was found by the least square regression of the data up to the P_m, the maximum antibiotic titer, with using maximum cell concentration. The yield factors; $Y_{x/s}$, $Y_{p/s}$ and $Y_{p/x}$, were also calculated based on the substrate glucose in each run and represented with their highest values. Initial glucose amount was taken into account for the evaluations of $Y_{x/s}$ and $Y_{p/s}$ rather than the consumed glucose.

Effect of Initial D-glucose Concentration

D-glucose concentration in the broth previously described was changed as 10, 15, 25, 40, 60, 70, 80, 90, 120 and 150 gL^{-1} keeping the other components fixed and results were presented in Figures 1-3.

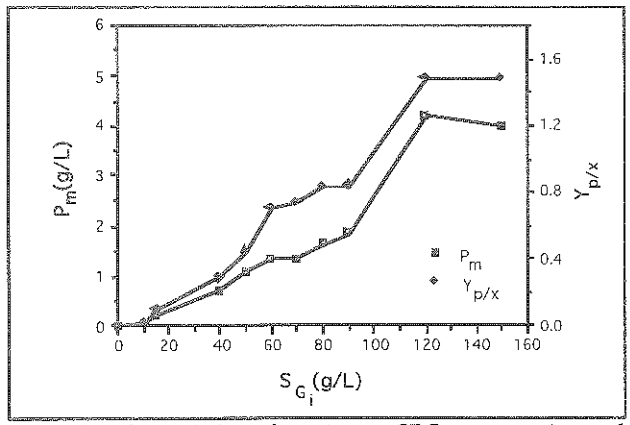

Figure 1. The variation of maximum CPC concentration and specific antibiotic yield by initial D-glucose concentration (P_m: maximum antibiotic concentration attained in the medium, gL^{-1}; $Y_{p/x}$: specific product yield of CPC, g CPC produced g^{-1} maximum microorganism attained; S_{Gi}: initial concentration of D-glucose in the medium, gL^{-1}).

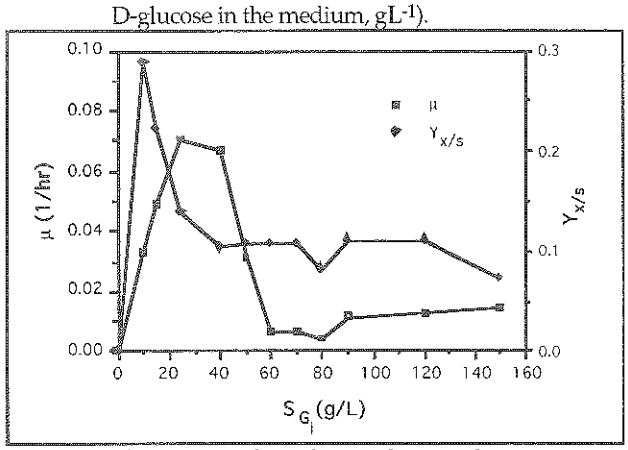

Figure 2. The variation of specific growth rate and microorganism yield by initial D-glucose concentration (μ: specific growth rate of the culture, h^{-1}; $Y_{x/s}$: yield factor for the cell, g microorganism produced g^{-1} initial glucose; S_{Gi}: initial concentration of D-glucose in the medium, gL^{-1}).

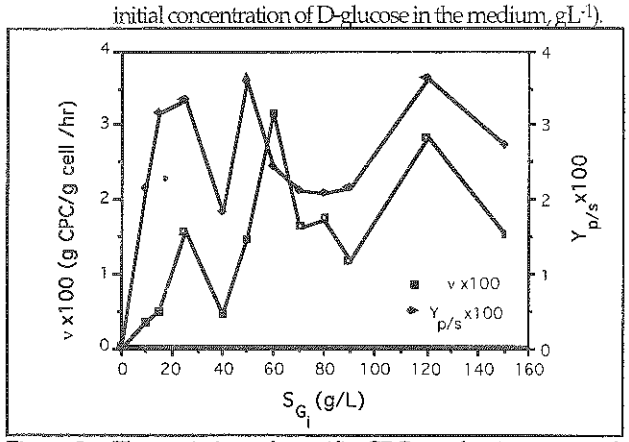

Figure 3. The variation of specific CPC production rate and antibiotic yield by initial D-glucose concentration (ν: specific production rate of CPC, g CPC produced L^{-1} h^{-1}; $Y_{p/s}$: yield factor for the antibiotic, g CPC produced g^{-1} initial glucose; S_{Gi}: initial concentration of D-glucose in the medium, gL^{-1}).

In Figure 1, a gradual increase was observed both for maximum CPC concentration, P_m, and specific product yield, $Y_{p/x}$, with increased initial glucose concentration; finally reaching to 4.2 gL^{-1} and 1.49 g CPC produced g^{-1} maximum microorganism attained, respectively. The increasing trend was almost identical for P_m and $Y_{p/x}$ containing local decreases and higher glucose concentrations definitely favored synthesis of the antibiotic CPC rather than growth. The maximum antibiotic concentration attained as 4.2 gL^{-1} was lower than 4.63 gL^{-1} obtained for airlift tower-loop reactors in literature studies[9].

In Figure 2, the maximum microorganism yield, $Y_{x/s}$, and specific growth rate, μ, were realized as 0.288 g microorganism produced g^{-1} glucose consumed and 0.0707 h^{-1}, respectively, at 10 gL^{-1} and 25 gL^{-1} of glucose concentrations, however after these peak values both parameters decreased due to inhibition effect of higher glucose concentration. Nevertheless, this substrate inhibition on both microorganism specific growth rate and microorganism yield certainly supported the CPC synthesis mechanism by limiting the metabolic activities for cell growth. However, for specific growth rate this substrate inhibition could not enforce zero growth, because after 80 gL^{-1} of glucose μ started to ascend again, being not fitted to any inhibition model in literature. Comparatively low values of cell yield , (<0.3), was due to large amounts of unused glucose in fermentation medium.

Figure 3 shows oscillatory behaviors of specific production rate, ν, and product yield, $Y_{p/s}$, with initial glucose concentration. The graphs of $Y_{p/s}$ and ν were almost the projected forms of each other showing the identical trends against inhibiting glucose levels. Increasing glucose concentration caused successive drops and rises in both ν and $Y_{p/s}$, but the microorganism could possibly alter its cell metabolism to cancel this inhibition effect. Maximum $Y_{p/s}$ and ν were realized as 3.61×10^{-2} g CPC produced g^{-1} glucose consumed and 3.15×10^{-2} g CPC g^{-1} cell h^{-1}, respectively, at 50 gL^{-1} - 60 gL^{-1} of glucose concentration, where the latter was much greater than experienced in literature[10].

Effect of Initial Ammonium Sulfate Concentration

Ammonium sulfate concentration in the broth previously described was changed as 7.5, 10, 15, 20, 25, 30, 40, 60 and 100 gL^{-1} keeping the other components fixed and results were presented in Figures 4-6.

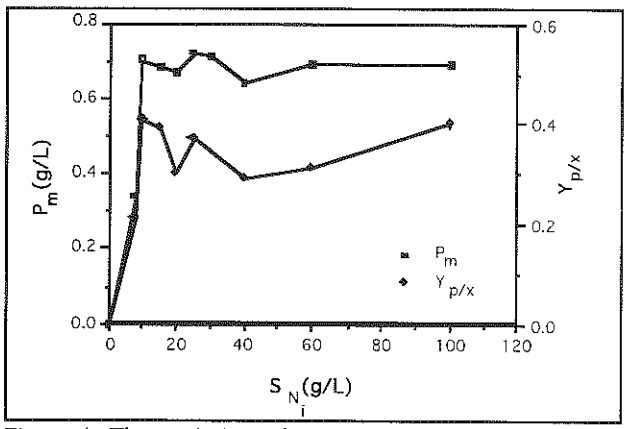

Figure 4. The variation of maximum CPC concentration and specific antibiotic yield by initial ammonium sulfate concentration (P_m: maximum antibiotic concentration attained in the medium, gL^{-1}; $Y_{p/x}$: specific product yield of CPC, g CPC produced g^{-1} maximum microorganism attained; S_{Ni}: initial concentration of ammonium sulfate in the medium, gL^{-1}).

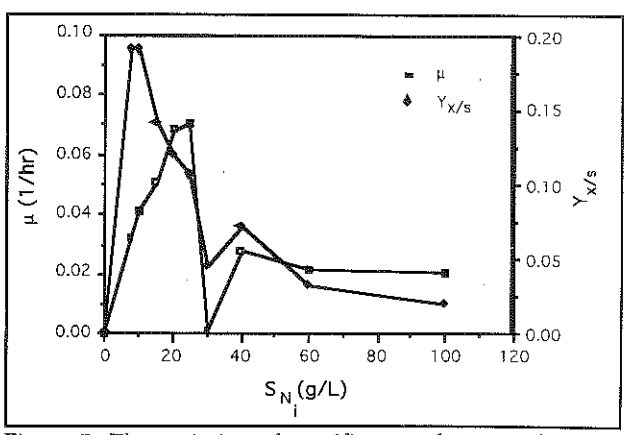

Figure 5. The variation of specific growth rate and microorganism yield by initial ammonium sulfate concentration (μ: specific growth rate of the culture, h^{-1}; $Y_{x/s}$: yield factor for the cell, g microorganism produced g^{-1} initial glucose; S_{Ni}: initial concentration of ammonium sulfate in the medium, gL^{-1}).

Figure 4 shows mutual increase of maximum CPC concentration, P_m, and specific product yield, $Y_{p/x}$, up to 10 gL^{-1} of ammonium sulfate concentration. After this concentration P_m was fluctuated around

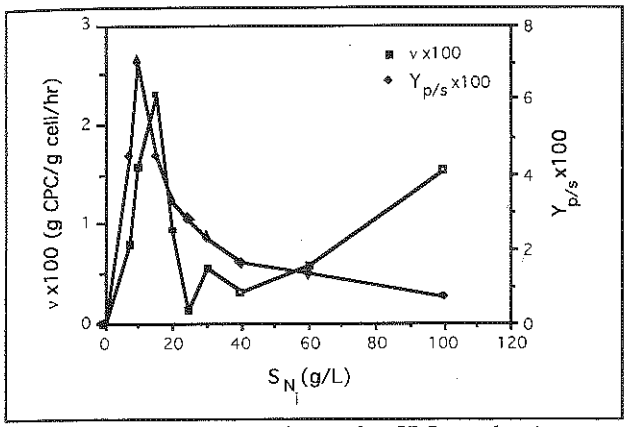

Figure 6. The variation of specific CPC production rate and antibiotic yield by initial ammonium sulfate concentration (v: specific production rate of CPC, g CPC produced L^{-1} h^{-1}; $Y_{p/s}$: yield factor for the antibiotic, g CPC produced g^{-1} initial glucose; S_{Ni}: initial concentration of ammonium sulfate in the medium, gL^{-1}).

0.7 gL^{-1} while $Y_{p/x}$ dropped but having a rise at higher ammonium sulfate concentrations. This final rise was due to decreased maximum microorganism concentration rather than an increase in CPC concentration (data not shown). Again, as is the case in Figure 1, P_m and $Y_{p/x}$ curves were almost identical but due to relatively low level of D-glucose in the broth (30 gL^{-1}), P_m was not realized as much as of higher D-glucose concentrations.

In Figure 5, specific growth rate, μ, was almost pinned down around 30 gL^{-1} of ammonium sulfate, however like in the glucose inhibition case (Figure 2) the culture accomplished necessary metabolic modifications to propagate again. Thus this growth trend did not fit into any type of inhibition model in literature. μ was maximized as 0.07 h^{-1} which were almost the same for glucose studies. Ideal microorganism yield was found in the range 7.5 - 10 gL^{-1}, however increasing ammonium sulfate concentration caused inefficient use of glucose source.

Figure 6 did not present an oscillatory behavior as observed in Figure 3 for increasing levels of glucose. Instead, specific production rate and antibiotic yield had maximums as 2.30x 10^{-2} g CPC g^{-1} cell h^{-1} and 7.0x10^{-2} g CPC produced g^{-1} glucose consumed at 15 gL^{-1} and 10 gL^{-1} of ammonium sulfate, respectively. Like in Figure 5, v decreased to the minimum value at 30 gL^{-1} of ammonium sulfate, rising afterwards. However this rise could be only attributed to decrease in maximum microorganism concentration of the fermentation medium (data not shown).

CONCLUSION

Highly differentiating and homogeneously growing culture of *Cephalosporium acremonium* (ATCC 14625) was employed for the production of cephalosporin C (CPC) and the effects of high initial D-glucose and ammonium sulfate concentrations were investigated. Maximum CPC concentration, P_m and specific product yield, $Y_{p/x}$ increased gradually with increasing glucose concentration where synthesis of the CPC was more favored rather than growth at higher glucose concentrations. Both microorganism yield, $Y_{x/s}$, and specific growth rate, μ, decreased due to inhibition effect at higher glucose levels, however, for μ zero growth was never reached with substrate inhibition since after 80 gL^{-1} glucose concentration μ started to increase again which was impossible to explain by any inhibition model existing in literature. Specific production rate, v, and product yield, $Y_{p/s}$, showed almost identical trends against inhibiting glucose levels where oscillatory behaviors were observed which were the projected forms of each other. In spite of successive drops and rises in both v and $Y_{p/s}$, the microorganism seemed to be capable of altering its cell metabolism against substrate inhibition.

Maximum CPC concentration and specific product yield showed similar increases with increasing ammonium sulfate concentration. As in the glucose case, specific growth rate dropped around 30 gL^{-1} ammonium sulfate, however, the microorganism again accomplished necessary metabolic modifications to propagate, again, by no means fitting into any type of inhibition model in literature. In ammonium sulfate case, specific production rate and antibiotic yield values did not present an oscillatory behavior as in glucose. v decreased to a minimum value at 30 gL^{-1} ammonium sulfate rising afterwards which could only be explained by the decrease in microorganism concentration.

Acknowledgments

We thank Glaxo Pharmaceutical Industry of UK for kindly providing the culture. This research was partially supported by Hacettepe University Research Fund with research grant # 84-01-010-59.

NOMENCLATURE

CPC	:	Cephalosporin C
P_m	:	Maximum antibiotic concentration attained in the medium (gL^{-1})
S_{Gi}	:	Initial concentration of D-glucose in the medium (gL^{-1})
S_{Ni}	:	Initial concentration of ammonium sulfate in the medium (gL^{-1})
t	:	Time elapsed during the fermentation (h)
X	:	Microorganism concentration on dry base (g cell L^{-1})
$Y_{p/s}$	:	Yield factor for the antibiotic (g CPC produced g^{-1} initial glucose)
$Y_{p/x}$	:	Specific product yield of CPC (g CPC produced g^{-1} maximum microorganism attained)
$Y_{x/s}$	:	Yield factor for the cell (g microorganism produced g^{-1} initial glucose)
μ	:	Specific growth rate of the culture (h^{-1})
ν	:	Specific production rate of CPC (g CPC produced L^{-1} h^{-1})

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