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Effect of Cultivation Scale and Shear Stress on Cell Growth and Oxytetracycline Production by *Streptomyces rimosus* in Semi-Defined Medium

Hoda Omar[1], Elsayed A. Elsayed[2,3], Ahmed A. Kenawy[4,5], Mohamed A. Wadaan[2] and Hesham A. El Enshasy[5,6]*

[1]Microbiology Department, Faculty of Pharmacy, Alexandria University, Alexandria, Egypt.
[2]Bioproducts Research Chair, Zoology Department, Faculty of Science, King Saud University, Riyadh, Saudi Arabia.
[3]Natural and Microbial Product Department, National Research Centre, Dokki, Cairo, Egypt.
[4]Faculty of Forestry, The University of British Columbia, Vancouver, BC, Canada
[5]Genetic Engineering and Biotechnology Research Institute, City for Scientific Research andTechnology Applications (CSAT), New Burg Al Arab, Alexandria, Egypt.
[6]Institute of Bioproduct Development (IBD), Universiti Teknologi Malaysia (UTM), 81310 Skudai, Johor, Malaysia.

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This study was focused on the investigation of the kinetics of cell growth and oxytetracycline (OTC) production during the cultivation of *Streptomyces rimosus* in submerged culture in different scales using semi-defined cultivation medium. In shake flask cultures, the volumetric and specific antibiotic production, reachedafter 108 h, were 170 mg/L and 37.9 mg/g, respectively. Further increases in volumetric and specific OTC productions was achieved upon transferring the process to stirred tank bioreactor and studying the effect of agitation speed on the kinetics of cell growth and antibiotic production. Of different agitation speeds applied ranging between 250 and 750 rpm, the maximal cell growth of 8.15 g/L and volumetric OTC production of 410 mg/L were obtained in 500 rpm agitated culture. Further increase in agitation speed, up to 750 rpm, decreased the biomass by about 40% and the volumetric OTC production by 29%.

Key words: *Streptomyces rimosus*, oxytetracycline, scaling up, shear stress, submerged culture.

Oxytetracycline or 5-hydroxytetracycline (CAS# 79572) is a polyketide antibiotic with the chemical structure $C_{22}H_{24}N_2O_9$ and average molecular weight of 460.434 (Fig. 1). This antibiotic was discovered by Pfizer laboratories in the late 1940s as secondary metabolite derived from soil actinomycetes *Streptomyces rimosus*. It is the second antibiotic discovered from the tetracyclines after the 7-chlorotetracycline (the well-known antibiotic family of a wide range of applications). The antimicrobial activities of oxytetracycline (OTC) are based on its ability to inhibit protein synthesis and cell growth (elongation) by binding the 30S ribosomal subunit and thus preventing the binding between the aminoacyl tRNA and the A site of the ribosomes. The high antimicrobial activities of OTC are also attributed to its lipophilic character and subsequently its high diffusion ability to pass through the cell membrane by simple passive diffusion through the porin channels in the bacterial membranes. Based on its wide spectrum activities against large number of bacteria such as *Diplococcus pneumonia, Escherichia*

* To whom all correspondence should be addressed.
Tel.: +60-75532595; Fax: +60-75532595;
E-mail: henshasy@ibd.utm.my

coli, *Mycoplasma pneumonia* and *Haemophilus influenzae*, OTC is commonly used in the treatment of different human and animal microbial infectious diseases[1]. In addition, this antibiotic is one of the few antibiotics which are still approved for non-medical applications as animal feed additives as well as a biological control agent for plant diseases and become one of the important metabolites in wellness industries[2-3]. These applications increase the demand on the continuous development and improvement of the production of this antibiotic in large scale. However, the industrial production of this antibiotic is carried out using limited numbers of strains such as *Streptomyces rimosus*, *Str. viridifuciens*, *Str. varsoviensis* and other non-fully identified *Streptomyces* spp.[4-6]. However, many research reports were published on the production of oxytetracycline in submerged culture[7], solid state fermentation[8,9], immobilized cells in different matrices[5,10,11], as well as in mixed culture systems[12,13]. However, most of the previous published works was carried out mainly by using complex culture medium and was performed on small scale. Accordingly, the present study was designed to examine the production of OTC in shake flask and in bioreactor levels. The cultivations were carried out in a semi-defined medium containing glucose as main carbon source. The kinetics of cell growth and antibiotic production in shake flask and in bioreactor operated at different agitation speeds were investigated. This study will help to understand the effect of hydrodynamic mixing on the kinetics of cell growth and OTC production in bioreactor level which is one of the main considered factors for bioprocess industrialization.

Fig. 1. Chemical structure of oxytetracycline

MATERIALS AND METHODS

Microorganisms

Streptomyces rimosus sub sp. *rimosus* DSMZ 41439 used in the present study was initially obtained as a lyophilized culture from the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany. This strain was first activated in ISP2 broth and was subsequently cultivated on ISP2 agar medium for 10 days at 28°C. The obtained spores were collected using glycerol solution 50%, maintained in cryovials (Nalgen, USA) at -80°C as cell bank for subsequent use. The viability of the deep-frozen cultures was periodically controlled by plating onto ISP2 agar having the following composition g/L: malt extract, 10; yeast extract, 4; glucose, 4; agar, 20. The pH of the medium was adjusted to 7.0±0.2 before sterilization by autoclaving. The test organism for oxytetracycline bioactivity determination was *Bacillus subtilis* NRRL-B543. This strain was obtained from Agriculture Research Service (NRRL) culture collection (Peoria, IL, USA).

Cultivation conditions

Inoculum for both shake flask and bioreactor was first prepared by inoculating 0.5 mL of spore suspension obtained from the frozen glycerin culture to a 250 mL Erlenmeyer flask of 50 mL working volume using ISP2 broth. The inoculated flasks were incubated on a rotary shaker (Innova 44, Eppendort, Germany) at 28°C and 200 rpm for 48 hours. The obtained vegetative cells were used to inoculate the production medium for both shake flask and bioreactor in a concentration of 5% v/v. The OTC production medium had the following composition (g/L): glucose, 30.0; yeast extract, 5.0; $(NH_3)_2SO_4$, 3.0; KH_2PO_4, 2.0; K_2HPO_4, 1.0; $MgSO_4.7H_2O$, 0.5. Glucose was sterilized separately at 110°C for 20 min., and was added to the medium before inoculation. The rest of medium components were sterilized together at 121°C for 15 min. For bioreactor culture, sterilization time was 30 min. In case of OTC production in shake flask culture, 250 mL Erlenmeyer flasks with a working volume of 50 mL were incubated on a rotary shaker (Innova 44, Eppendorf, Germany) at 28°C, 200 rpm, and shaking eccentricity of 20 mm. Bioreactor cultivations were carried out using 2-L stirred tank bioreactor (Eppendorf, NJ, USA) with a working volume of 1.5 L. The stirrer was equipped with two

6-bladed Rushton turbine impellers ($d_{i(\text{impeller diameter})}$ = 52 mm, $d_{t\ (\text{tank diameter})}$ = 125 mm, d_i/d_t = 0.416), agitation was adjusted at constant speed of different values 250, 500, and 750 rpm according to the condition of the experiment. Aeration was performed using filtered sterile air at a rate of 1 v/v/min. The pH was determined throughout the cultivation using *in situ* sterilizable pH electrode (Mittler Toledo, Switzerland) attached to the control panel of the bioreactor. The dissolved oxygen concentration (DO) was analyzed using *in situ* polarographic electrode (Ingold, Mittler Toledo, Switzerland). During cultivation, foam was suppressed by the addition of the antifoam agent Struktul (Th. Goldschmidt AG, Essen, Germany).

Sample preparation and cell dry weight determination

Samples in the form of 2 flasks of 50 mL broth each (in case of shake flask cultivation) or 10 mL broth in case of bioreactor culture were taken at different time intervals during the cultivation and were collected in pre-weighed 50 mL sterile centrifugation tube (Falcon, USA). Samples were centrifuged immediately at 3,000 rpm and 10°C for 30 min. The supernatant was frozen at -20°C for further analysis, whereas the centrifuged cells were washed twice by distilled water, followed by centrifugation. The centrifuged tubes were dried in vacuum oven at 40°C until constant weight was achieved for cell dry weight determination.

Analysis

Glucose determination

Glucose concentration was determined using glucose kit (Diamond Diagnostics, Egypt). This method is based on the glucose conversion to gluconic acid using glucose oxidase-glucose peroxidase enzyme systems. The intensity of the developed color was determined at 500 nm using spectrophotometer (Pharmacia Biotech., Cambridge, England).

Determination of oxytetracycline

The oxytetracycline concentration in the fermentation broth was quantitatively determined by the agar plate diffusion method. A biological standard curve was drawn between the logarithm of different concentrations of standard oxytetracycline and the diameter of the inhibition zone of the susceptible bacterial strain *Bacillus subtilis* NRRL-B543.

RESULTS

Cell growth and OTC production in shake flask culture

The cell growth, glucose consumption and OTC production kinetics in shake flask cultivation at 200 rpm for 160 hours are represented in Fig.2. As shown, cells grew exponentially without lag phase with a growth rate of 0.05 g/L/h and reached a maximal biomass of 4.65 g/L after 96 h. After that time, cell concentration was almost the same for the rest of the cultivation time. During the active growth phase, glucose concentration decreased in culture as a result of active glucose consumption with a rate of 0.145 g/L/h during the first 84 hours. As cells entered the stationary phase, glucose consumption in culture was as low as 0.02 g/L/h which may be just used for maintenance energy. On the other hand, OTC was produced in culture parallel to cell growth immediately after the inoculation and accumulated in culture with a production rate of 1.57 mg/L/h. After 108 h, OTC reached its maximal value of about 170 mg/L and kept more or less constant for the rest of the cultivation time. The absence of lag phase in this culture was due to the good inoculum quality as it was in the form of vegetative cells previously grown for 48 hours in complex medium. It is also worthy to note that, the pH of the culture dropped from about 6.8 to 5.8 during the first 24 hours and increased gradually thereafter reaching 7.5 as cells entered the stationary phase (after 96 hours) and kept more or less constant for the rest of the cultivation time.

Bioreactor cultivations

The cell growth and OTC production were also studied in bioreactor batch culture under different agitation speeds. All cultivations were carried out using the same medium composition, inoculum size, temperature and initial pH as in shake flask cultures. Three experiments were performed using 2-L laboratory scale stirred tank bioreactor with 1.5-L working volume operated under the same aeration rate of 1 v/v/min but with different agitation speeds of 250 (low agitation), 500 (medium agitation), and 750 rpm (high agitation).

Cell growth and OTC production at low agitation speed (250 rpm)

Fig.3 demonstrates the results of batch cultivation of *Str. rimosus* cultivated at constant

agitation speed of 250 rpm. As shown, immediately after inoculation, cells grew exponentially with a growth rate of 0.064 g/L/h reaching a maximal biomass production of 6.35 g/L after 108 h. In parallel to cell growth, OTC was produced without any significant lag phase and accumulated continuously in the culture with a rate of 2.19 mg/L/h. The maximal antibiotic production of 215 mg/L (about 26% higher than that obtained in shake flask culture) was obtained after 120 hours. During the active growth phase, glucose was consumed in culture as a function of cell activity with a rate of 0.185 g/L/h. Once cells started to enter the stationary phase (after 96h), the glucose consumption rate decreased to 0.037 g/L/h. During the exponential growth phase, the DO decreased gradually in culture due to cell growth and reached about 30% saturation after about 80 h. As cells entered the stationary phase, the DO increased gradually and reached about 50% saturation at the end of the cultivation time. Additionally, the pH of the culture dropped from its initial value (pH 7.0) to 5.8 during the first 48 h and increased gradually thereafter reaching about 8.0 during the late exponential growth phase (about 80 h) and kept more or less constant for the rest of cultivation time.

Cell growth and OTC production at medium agitation speed (500 rpm)

In this experiment, cultivation was carried out at medium agitation speed of 500 rpm. In this case, more biomass was formed compared to the lower agitation speed culture. As shown in Fig. 4, the maximal biomass formed was about 8.15 g/L (about 28% higher than that obtained in the low-speed agitated culture). In addition, this maximal biomass value was obtained after only 84 h (compared to 108 in the case of low-speed agitated culture). During the active growth phase, glucose was consumed in culture with a rate of 0.285 g/L/h (almost 54% higher compared to culture at 250 rpm). After this phase, glucose consumption rate decreased only to 0.063 g/L/h which is also higher compared to 250 rpm culture during the stationary phase. Compared to the low agitation speed, OTC production reached 410 mg/L after 120 h and kept more or less constant for the rest of the cultivation.

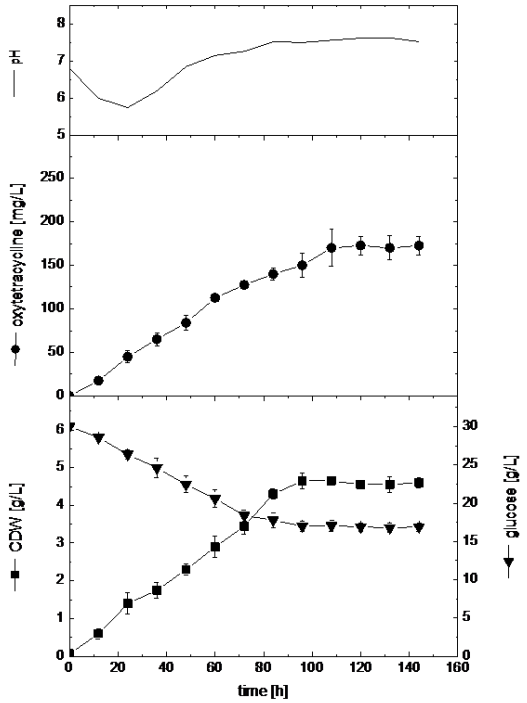

Fig. 2. Cell growth, glucose consumption, oxytetracycline production, and pH profile during batch cultivation of *Str. rimosus* in shake flasks at 200 rpm and 28 °C

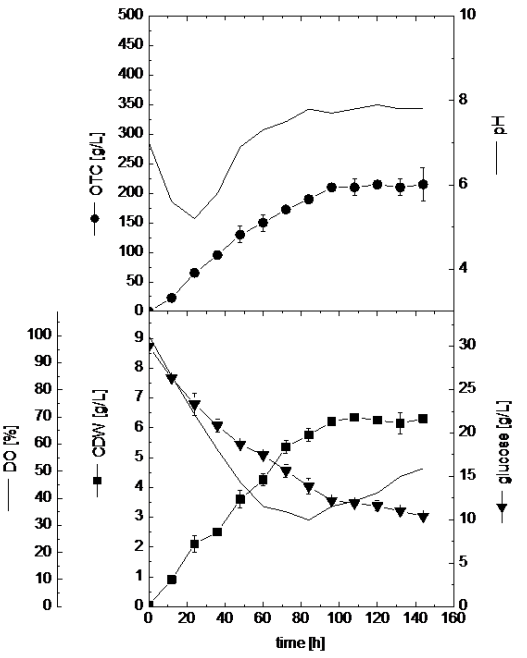

Fig. 3. Cell growth, glucose consumption, oxytetracycline production, and pH profile during batch cultivation of *Str. rimosus* in stirred tank bioreactor at 250 rpm, aeration rate 1/v/v/min. and 28°C.

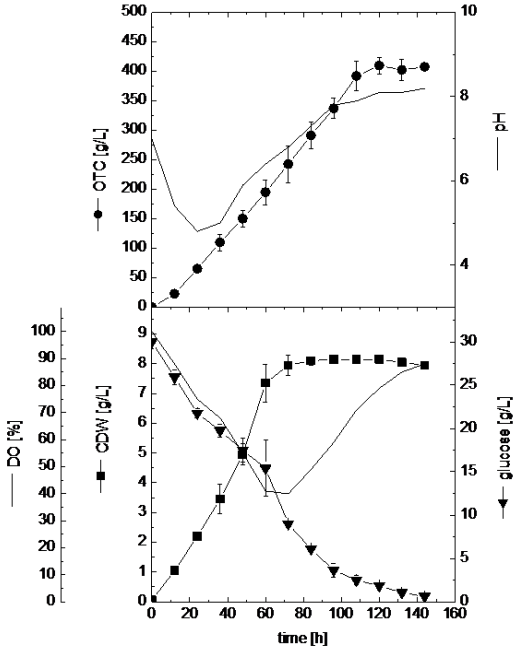

Fig. 4. Cell growth, glucose consumption, oxytetracycline production, and pH profile during batch cultivation of *Str. rimosus* in stirred tank bioreactor at 500 rpm, aeration rate 1/v/v/min. and 28°C

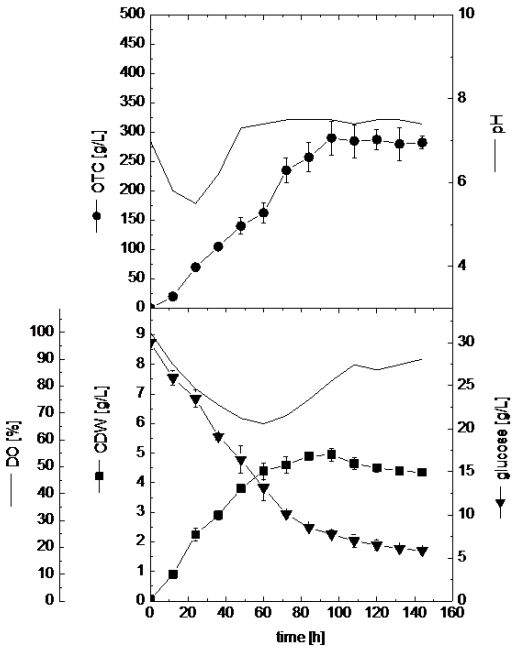

Fig. 5. Cell growth, glucose consumption, oxytetracycline production, and pH profile during batch cultivation of *Str. rimosus* in stirred tank bioreactor at 750 rpm, aeration rate 1/v/v/min. and 28°C

This value was about 91% higher compared to the corresponding batch culture at agitation speed of 250 rpm. Comparable to 250 rpm-agitated culture, pH dropped significantly during the early growth phase reaching 4.9 after 48h and then increased gradually reaching about 8 at the end of cultivation time. Moreover, DO in the culture decreased during the active growth phase (exponential growth) reaching about 40% saturation and then increased gradually once the cells entered the stationary phase up to almost 85% saturation (after 144 h).

Cell growth and OTC production at high agitation speed (750 rpm)

Cultivation at high agitation speed yielded the lowest biomass production among other cultivation, where the maximal biomass produced in this culture was about 4.95 g/L after 84 h (Fig. 5). This value was almost the same as in shake flask culture. During growth phase, like in other cultures, glucose was consumed with a rate of 0.255 g/L/h and decreased to 0.045 g/L/h as cells entered the stationary phase. For antibiotic production, the maximal value of OTC production of 290 mg/L was obtained after 96 h and kept more or less constant for the rest of the cultivation time. On the other hand, the drop in pH and DO was not so large as the other bioreactor cultures. The pH dropped from its initial value (7.0) to only 5.8 after 48 h, then increased gradually reaching about 7.4 after 72 h and kept more or less constant for the rest of the cultivation time. For DO, the lowest value of about 65% saturation was obtained after 60 h as well, and increased gradually thereafter reaching more than 90% saturation at the end of the cultivation.

DISCUSSION

The present investigation clearly demonstrates the differences in the kinetics of cell growth and OTC production parameters based on the production scale and the agitation intensity during bioreactor cultivations (Table 1). Generally, bioreactor cultures are characterized by higher biomass production compared to shake flask ones. The significant increase in the growth in bioreactor cultures is due to the better aeration and agitation conditions which support the better mixing between

air/liquid phases and thus increase oxygen solubility in the culture. The higher growth rate and biomass production were expected, since it was also reported that scaling up the process from shake flask to stirred tank bioreactor promotes higher cell growth of aerobic filamentous microorganisms under non-limiting substrate conditions such as in the case of cyclosporine production by *Tolypocladium inflatum*[14] and tylosin production by *Streptomyces fradiae*[15]. On the other hand, the volumetric OTC production was higher in all bioreactor cultures under study. The maximal value of OTC of 410 mg/L was obtained in 500 rpm agitated culture. This value was almost 140% higher than the value obtained in shake flask and higher than other bioreactor cultures by about 91 and 41% for 250 and 750rpm agitated cultures, respectively. In general, in case of filamentous microorganisms, growth morphology is an important factor which governs the cell productivity. In many processes, growth in the micropellet form not larger than 400 µm diameter is the most favorable morphology for the production of metabolites from aerobic filamentous microorganisms[16,17]. In this study, growth morphology in all cultivation scales was in the form of small micropellet and did not exceed the critical pellet diameter for aerobic microorganisms (i.e. 400 µm). This growth morphology was found to be independent on shear stress. Thus, the differences in cell productivities were not related to growth morphology in the current study. For the better understanding of cell performance and productivity in different cultures, the yield coefficient $Y_{p/x}$ was calculated in all cultures. It was clearly observed that the cell productivity was highly dependent

Table 1. Kinetic parameters for cell growth, OTC production and glucose consumption during *Str. rimosus* cultivation in shake flask and bioreactor under different agitation speeds

Parameter	Type of cultivation vessel			
	Shake Flask		Bioreactor	
Cultivation Parameters				
Agitation speed (rpm)	200	250	500	750
Initial pH	6.8	7.0		
Cultivation temperature	28 °C			
Working volume [mL]	50	1500		
Growth and substrate consumption parameters				
$X_{max\text{-}conc.}$ [g/L]	4.65	6.35	8.15	4.95
$X_{max\text{-}time.}$ [h]	96	108	84	84
dx/dt [g/L/h]	0.05	0.06401	0.096	0.058
m[1/h]	0.029	0.022	0.023	0.024
$Q_{s\ (log\ phase)}$ [g/L/h]	-0.145	-0.185	-0.285	-0.255
$Q_{s\ (stat\ phase)}$ [g/L/h]	-0.037	-0.0375	-0.063	-0.045
Antibiotic production parameters				
$P_{max\text{-}conc.}$ [mg/L]	170	215	410	290
$P_{max\text{-}time}$ [h]	108	120	120	96
Q_p [mg/L/h]	1.57	2.19	3.63	3.02
Yield coefficients				
$Y_{x/s}$ [g/g]	0.358	0.356	0.339	0.228
$Y_{p/x}$ [mg/g]	37.90	33.87	50.30	61.29
$Y_{p/s}$ [mg/g]	13.17	11.79	14.56	13.03

Abbreviations: $X_{max\text{-}conc.}$, maximal cell dry weight; $X_{max\text{-}time.}$ time of maximal cell dry weight; dx/dt, cell growth rate; m, specific growth rate; $Q_{s\ (log\ phase)}$, glucose consumption rate during the log phase; $Q_{s\ (stat\ phase)}$, glucose consumption rate during the stationary phase; $P_{max\text{-}conc.}$, maximal OTC production; $P_{max\text{-}time}$, time of maximal OTC production; Q_p, OTC production rate; $Y_{x/s}$, g biomass per g glucose consumed; $Y_{p/x}$, mg OTC produced per g biomass; $Y_{p/s}$, mg OTC per g glucose consumed.

on the agitation speed. Thus, the higher observed volumetric production at 500 rpm compared to the lower agitation speed was attributed in part to the increase in cell productivity and not only due to the increase of biomass (Table 1). Increasing the agitation speed up to 750 rpm resulted in a further increase in cell productivity (from 50.30 to 61.26mg/g), however a concomitant decrease in biomass from 8.15 to 4.95 g/L was observed. Thus, we can conclude that the observed higher increase in OTC production at 500 rpm compared to 750 rpm was due to the increase in biomass production and not due to the increase in cell productivity. Comparing between these two cultivations (Fig. 4, 5), it can be clearly observed that the DO dropped to about 40% saturation during the growth phase in 500 rpm agitated culture whereas, the minimal DO reached in 750 agitated culture was 70% saturation and increased immediately after 60 hours (once cells are close to enter the stationary phase). Thus, we can conclude that higher agitation, which resulted in higher DO values in the culture, was not suitable for cell growth; otherwise it promoted antibiotic production and reduced the production time from 120 h to only 96 h. The phenomenon of the influence of dissolved oxygen in culture and the oxidative stress on microbial growth and antibiotic production was also reported by many authors. It was reported that tylosin production by *Str. fradiae* was severely reduced when DO fell below 25% saturation[18]. Other studies showed that the optimal DO value for antibiotic production by *Xenorhabdus nematophila* was 50% saturation[19]. However, the optimal DO for antibiotic production is not generic and is highly dependent on the type of the antibiotic and the producing strain.

CONCLUSIONS

The results obtained from shake flask and bioreactors operated under different agitation speeds demonstrate that cell growth and OTC production were higher in bioreactor cultures at all agitation speeds applied. Moreover, operation at medium agitation speed of 500 rpm was the optimal for the production of the antibiotic, which reached about 410 mg/L (about 240% higher than that obtained in shake flask cultures). The increase in cell growth and OTC production in bioreactor culture was supported by higher glucose consumption rate compared to shake flask cultures.

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