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Continuous Production of Oxytetracycline in Fluidized Bed Bioreactor by Immobilized Cells of *Streptomyces varsoviensis* MTCC-1537: Effect of Dilution and Glucose Loading Rates

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Abstract Oxytetracycline (OT) production using glutaraldehyde cross-linked calcium alginate immobilized cells of *Streptomyces varsoviensis* in continuous fluidized bed reactor (FBR) was investigated. Initially, batch experiments were carried in stirred tank reactor (STR) and FBR using calcium alginate immobilized cells. Higher OT production of 0.45 gm/L was achieved by FBR when compared with 0.33 g/L of OT in STR. All subsequent studies were carried out in continuous mode of operation in FBR. During 21 days of operation, effect of glucose concentration and different dilution rates were studied. A maximum of 0.75 g/L OT was achieved in the medium having 10 g/L of glucose concentration. The highest OT concentration of 0.92 g/L and the highest yield of OT with respect to biomass at 0.1713 g/g were obtained at the dilution rate of 0.25 day^{-1}.

Keywords Yield kinetics · Cross-linking · Antibiotics production · Glucose concentration · Continuous process · Stirred tank reactor

Introduction

Streptomyces species have been the focus of many researchers since they produce various therapeutically important antibiotics [1–4]. Oxytetracycline from *Streptomyces rimosus* in solid-state fermentations [5, 6] and in submerged fermentation [7] have been studied by various researchers. Immobilization offers several potential advantages to the fermentation systems over free cell systems from the process engineering standpoint, which include ease of handling and cell separation. The rationale for choosing whole cell immobilization is that it provides higher antibiotic yields after immobilization, higher operational stability, greater resistance to environmental disturbances, and lower effective operational costs [7–9].

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Fluidized bed reactors are advanced bioreactors used for the fermentative production of primary and secondary metabolites, and also, it has many advantages over stirred tank reactors (STR) and airlift reactors [10]. Continuous three-phase fluidized bed bioreactors promote intimate contact between solid, liquid, and gas. Earlier studies also indicated that high mass transfer rates and antibiotic production is possible from fluidized bed bioreactors. Liquid as the continuous phase has been used for the production of cephalosporin C [11], penicillin [12] and streptomycin [13], gentamicin [9] in fluidized bed reactor (FBR), and oxytetracycline in air bubbled reactor [7].

In the present study, oxytetracycline (OT) in a continuous FBR with immobilized cells of S*treptomyces varsoviensis* has been investigated. *S. varsoviensis* cells were immobilized in calcium alginate cross-linked with glutaraldehyde were used for the production of OT in a continuous fluidized bed reactor. The effect of different glucose concentration and different dilution rates were investigated to study the reactor performance with respect to antibiotic production.

Materials and Methods

Microorganism and Culture Conditions

The strain *S. varsoviensis*, NCIB-9522, MTCC-1537, used in the study was procured from Microbial Type Culture Collection (MTCC) Institute of Microbial Technology, Chandigarh, India. The culture was maintained on agar slants using actinomycetes agar medium (Hi Media Bombay). The growth medium contained (g/L): glucose 4, yeast extract 4, malt extract 10, $CaCO_3$ 2, and agar 20 at pH 7 and subcultured at monthly intervals. The composition of production medium consists of (g/L): glucose 30, corn steep liquor 9, $CaCO_3$ 2, ammonium sulfate 3.5, $MgSO_4{\cdot}7H_2O$ 0.1, and $CaCl_2$ 0.005 at pH 7.

Immobilization of Whole Cells

Cell suspension prepared from 5-day-old slants of *S. varsoviensis* was used to inoculate 50 mL of growth medium and incubated in orbital shaker at 170 rpm at 28±2°C. After 48 h, culture was centrifuged, washed with sterile distilled water, and the resultant cell pellet was used to prepare immobilized beads. Cells were immobilized using calcium alginate cross-linked with a glutaraldehyde entrapment method [14]. Sterilized solution of 3% sodium alginate and 100 μl glutaraldehyde was mixed with 0.06% of cells on dry cell weight basis (DCW, *w*/*v*) and mixed well to get uniform suspension. The above mixture was dropped into 0.2 M $CaCl_2$ solution using peristaltic pump (Watson and Marlow, UK). The immobilized beads having ~2- to 2.5-mm diameter were cured in 0.2 M $CaCl_2$ solution for 24 h and washed twice with sterile saline for further use.

Fluidized Bed Reactor Operation (Fig. 1)

The fluidized bed reactor was made of glass having dimensions of: upper diameter—9.8 cm, lower diameter—5.3 cm, total height—38 cm, working height—36.5 cm, and conical height of the bottom 5 cm containing a concentric draft tube of 58-cm height and 2-cm diameter. The working volume of the reactor was 1.0 L. The conical bottom of the reactor space is filled with steel beads of 2-mm diameter and the draft tube from the top was embedded within the steel beads. This kind of arrangement was applied to eliminate the use

of additional air sparger, as steel beads assist in the breaking of the larger air bubbles into fine air bubbles for smooth fluidization and for increasing the rate of oxygen mass transfer. The liquid feed was pumped into the reactor using a peristaltic pump. The reactor outlet was provided with a steel wire mesh to prevent the escape of immobilized beads from the reactor. The reactor was designed and fabricated at Bioengineering AG, Sagennarain Strasse 7, CH-8636 Wald, Switzerland. B. Brown (Bioastat B, Germany) Fermentor (1.5 L) with 1.0-L working volume was used for STR studies.

Three continuous runs designated as R1, R2, and R3 were performed using immobilized cells of *S. varsoviensis* for OT production for 21 days. Initially, FBR was operated in batch mode for 4 days to build up the biomass, followed by continuous operation for 21 days. In run R-1, the FBR was operated at 0.3-day^{-1} dilution rate with medium containing 15 g/L of glucose. In run R-2, the feed flow rate was fixed at 0.3 L/day, but the concentration of glucose in the medium was varied from 5 to 20 g/L with a step increment of 5 g/L. After batch mode of 4 days, FBR was fed with glucose at concentrations of 5, 10, and 15 g/L for 5 days and 20 g/L for 2 days. In run R-3, the concentration of glucose in the medium was fixed at 10 g/L, but the dilution rates were varied from 0.2 to 0.35 day^{-1}. After 4 days of batch mode, FBR was fed with medium at flow rates of 0.2 L/day for 5 days and 0.25, 0.3, and 0.35 L/day for 4 days each.

Analytical Methods

Samples were drawn under aseptic conditions for antibiotic assay, carbohydrate estimation, and pH. OT concentration was determined by plate diffusion assay using OT (Himedia) as standard and *Escherichia coli* as test organism [15]. Carbohydrate was estimated by anthrone method [16]. Biomass in the broth was estimated in 5 mL of sample after centrifugation at 8,000 rpm for 10 min and dried at 103 °C for 3 h. All the estimations were carried out in triplicates and the average was presented in the results. Biomass in the immobilized beads was analyzed by taking five beads for each sample. The beads were washed with distilled water and dissolved in 2% sodium hexametaphosphate to release the biomass entrapped in alginate. Cell pellet was collected by centrifugation and dried at 103 °C for 3 h [17].

Results

Batch Reactors (FBR and STR)

OT production with Ca-alginate entrapped *S. varsoviensis* was carried out in FBR and STR for 6 days in batch mode. Figure 2a, b shows OT production, biomass growth, and glucose utilization during 6 days of batch operation of FBR and STR. Maximum OT concentration of 0.45 g/L was observed on the fourth day and subsequently reduced to 0.3 g/L in FBR at the end of the sixth day. Increase in DCW of 9.96 g/L was observed on the fourth day and then decreased to 7.2 g/L at the end of the sixth day in FBR, whereas a maximum DCW of 9.41 g/L was recorded in STR. The carbohydrate utilization pattern was almost similar in FBR and STR; however, the OT production pattern and concentration of biomass showed significant difference in FBR when compared to STR. Maximum concentration of OT recorded in STR was 0.33 g/L, while 0.45 g/L was achieved in FBR, which was 27 % higher than STR.

OT production kinetics such as yield of biomass with respect to substrate ($Y_{X/S}$, mg/mg), yield of product with respect to substrate ($Y_{P/S}$, g/mg), and specific product formation of OT (SOF, $Y_{P/X}$, g/mg) were calculated with respect to dry cell weight of biomass in the reactor.

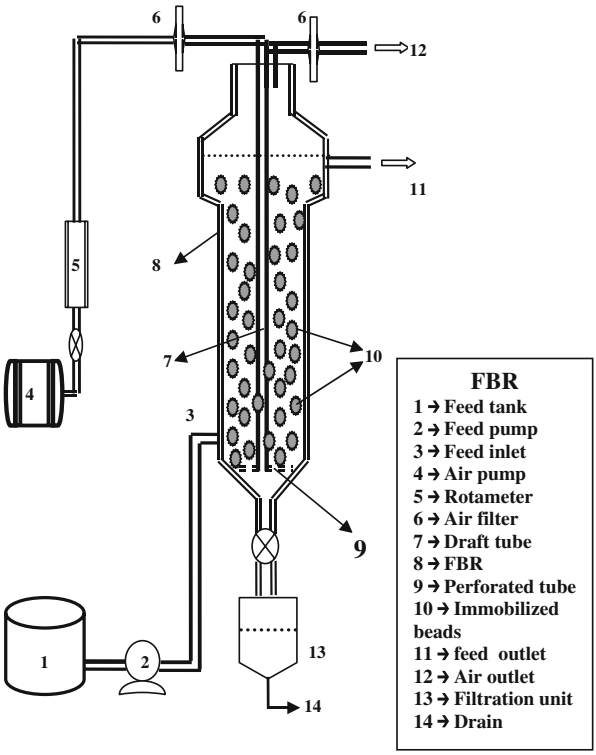


Fig. 1 Schematic process set up of fluidized bed bioreactor (*FBR*) for the production of oxytetracycline using *S. varsoviensis* with all accessories

Continuous Fluidized Bed Reactor

Continuous production of OT with Ca-alginate entrapped *S. varsoviensis* was carried out in FBR for 21 days (R1), and the OT concentration, biomass growth, and glucose utilization were shown in Fig. 3a. Production media having glucose concentration of 15 g/L was fed to FBR in continuous mode at a dilution rate of 0.3 day^{-1}. Maximum OT concentration of 0.63 g/L was recorded on day 14 and then the concentration decreased to 0.32 g/L at the end of reactor operation. DCW was continuously increased with the increase in OT. A maximum DCW of 6.9 g/L was observed on day 12 and maintained almost constantly at 6.3 g/L until the end of 21 days of operation. Sugar utilization was increased with the increase in OT concentration and biomass production. The decrease in sugar utilization after 17 days might be due to the decrease in biomass level. Increased utilization of sugar resulted in an increased production of OT and biomass growth in the fermentation system. In the present study, OT production was maintained at 0.67 g/L at optimum glucose concentration and flow rate for 21 days using immobilized cells of *S. varsoviensis*.

Effect of Glucose Feeding Rates on OT Productivity and Biomass Formation

Initially, FBR was operated in batch mode for 4 days to build up the biomass, followed by continuous operation for 21 days, and the data were shown in Fig. 3b. Glucose was fed to continuous FBR at the levels of 5, 10, and 15 g/L for time periods of 5 days each and 20 g/L for 2 days at the dilution rate of 0.3 day^{-1}. Increase in the feeding concentration of glucose

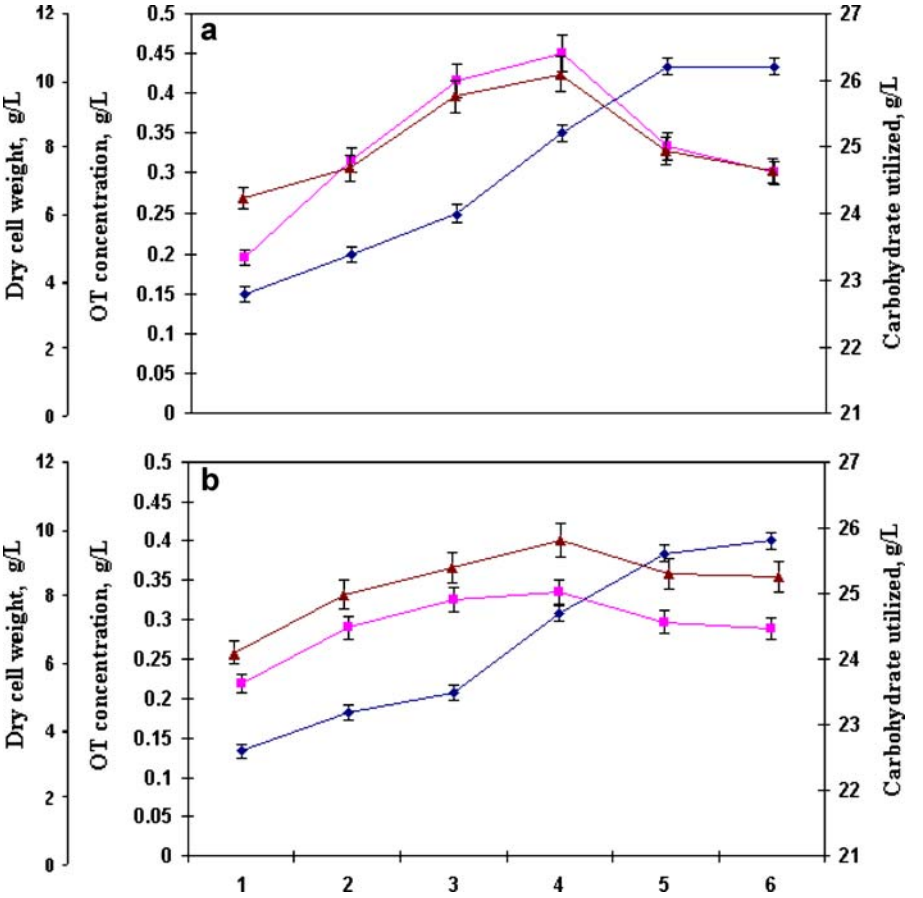
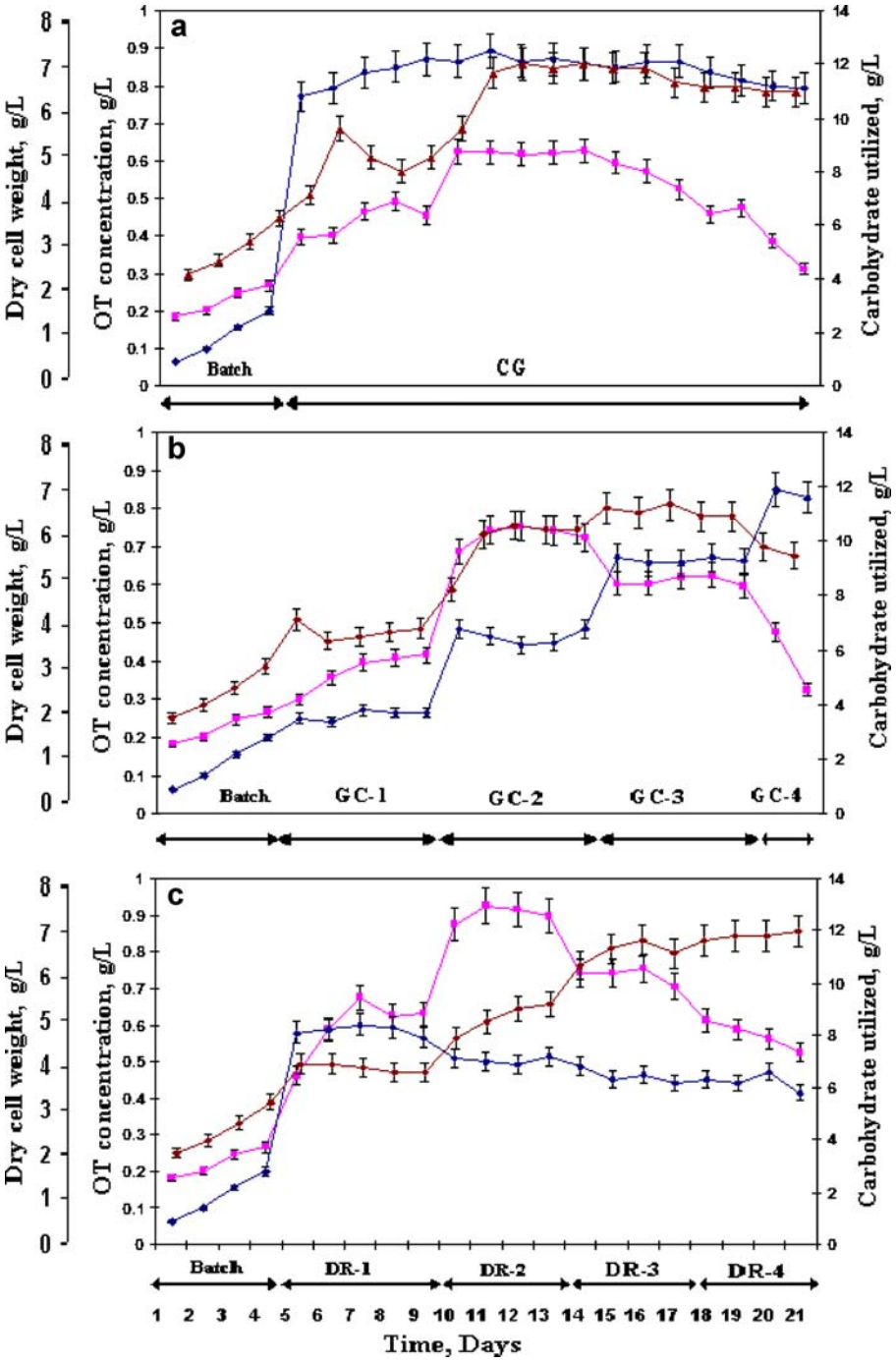


Fig. 2 Time profile of batch fluidized bed reactor (*FBR*) (**a**) and batch stirred tank reactor (*STR*) (**b**). Oxytetracycline (*OT*) concentration (*square*); dry cell weight (*DCW*, *triangle*); carbohydrate utilization (*diamond*). Standard deviation of DCW, OT, and carbohydrate utilization for FBR is 1.0123, 0.04862, and 1.2895 and for STR is 1.2895, 0.08925, and 1.2356, respectively

from 5 to 10 g/L increased OT production. Maximum OT production was observed at 0.41, 0.75, and 0.62 g/L with 5, 10, and 15 g/L of glucose, respectively. Feeding of high glucose concentration of 20 g/L decreased OT production to 0.47 g/L.

Effect of Different Dilution Rates on OT Production and Biomass Formation

The continuous FBR experiments from day 5 were initiated (R3) with a dilution rate of 0.2 day^{-1}, which was subsequently increased in steps to 0.25, 0.3, and 0.35 day^{-1}, and the results were shown in Fig. 3c. Each step was run under steady-state conditions by the adjustment of corresponding feed flow rates between each step. The steady-state conditions with different dilution rates were tracked by repeated sampling and measurement of OT, total biomass, and residual sugar concentrations in the reactor. The OT concentration was increased from 0.67 to 0.92 g/L with corresponding increase in the dilution from 0.2 to 0.25 day^{-1}, while further increase in the dilution rate of 0.35 day^{-1} decreased the OT


a
Dry cell weight, g/L
OT concentration, g/L
Carbohydrate utilized, g/L
Batch
CG
b
Batch
GC-1
GC-2
GC-3
GC-4
c
Batch
DR-1
DR-2
DR-3
DR-4
Time, Days

◀ **Fig. 3** Time profile of continuous fluidized bed reactor for oxytetracycline (*OT*) production under different operating conditions. In all operations, first 4 days was operated in batch mode followed by continuous operation. OT concentration (*square*); dry cell weight (*DCW*, *triangle*); glucose utilization (*diamond*). **a** 0.3-L/day constant medium flow rate with 15 g/L of constant glucose (*CG*) concentration. **b** 0.3-L/day constant medium flow rate with varied glucose concentrations (*GC*): GC-1, 5 g/L; GC-2, 10 g/L; GC-3, 15 g/L; GC-4, 20 g/L. **c** 15-g/L constant glucose concentration with varied dilution rates: DR-1, 0.2 h^{-1}; DR-2, 0.25 h^{-1}; DR-3, 0.3 h^{-1}; DR-4, 0.35 h^{-1}. Standard deviation for DCW, OT, and carbohydrate utilization at R1: 1.21, 0.1685, and 14.25; R2: 1.861, 0.213, and 3.1256; R3: 1.689, 0.1986, and 2.1258, respectively

concentration to around 0.5 g/L. Maximum OT production recorded with different dilution rates such as 0.2, 0.25, 0.3, and 0.35 day^{-1} were 0.67, 0.92, 0.75, and 0.59 g/L, respectively. SOF was increased from 0.153 to 0.171 g/L with increase in the dilution rated from 0.2 to 0.25 day^{-1}; further increase in dilution rate resulted in a decreased SOF.

Figure 4 shows the yield of OT ($Y_{P/S}$), biomass ($Y_{X/S}$), and SOF with respect to biomass ($Y_{P/X}$) in continuous FBR under different inlet glucose concentrations. The SOF was increased from 0.093 to 0.127 with increase in the glucose concentration from 5 to 10 g/L. Further increase in glucose concentration (20 g/L) decreased the SOF to 0.074. In contrast,

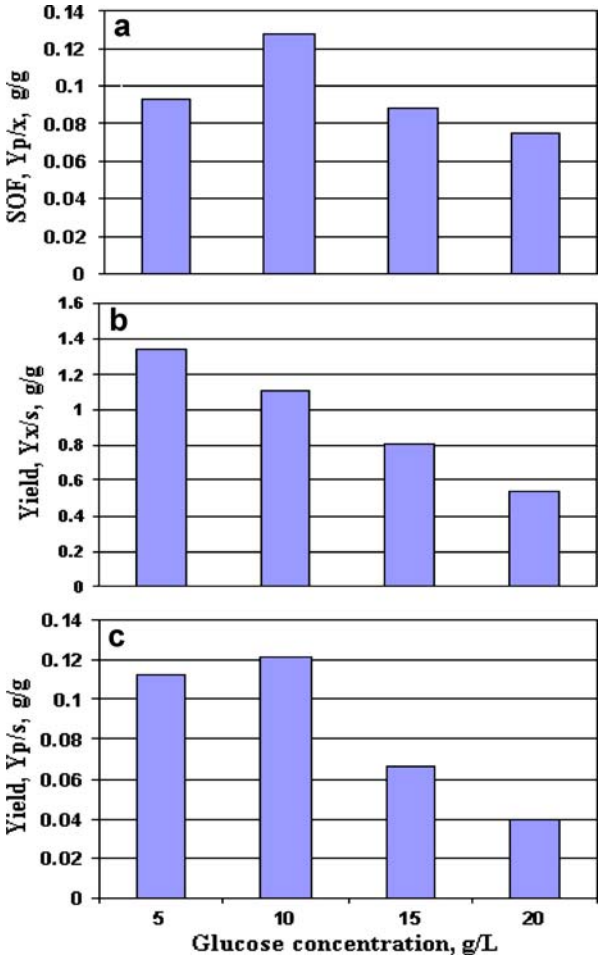


Fig. 4 Yield kinetics of OT in FBR under different glucose concentrations. **a** Specific OT formation (*SOF*), $Y_{P/X}$; **b** Biomass yield with substrate, $Y_{X/S}$; **c** Product yield with substrate, $Y_{P/S}$

yield of biomass and yield of OT with respect to substrate were decreased from 1.34 and 0.11 to 0.54 and 0.04 with increase in glucose concentration from 5 to 20 g/L.

Figure 5 shows the yield of OT ($Y_{P/S}$), biomass ($Y_{X/S}$) and specific OT formation with respect to biomass (SOF, $Y_{P/X}$) in continuous FBR under different dilution rates. The yields of biomass with respect to substrate ($Y_{X/S}$) were increased from 0.556 to 1.145 mg/mg with increase in the dilution rate from 0.2 to 0.3 day^{-1}. Maximum biomass production of 7.6 g/L was obtained at high dilution rate (D=0.35 day^{-1}), whereas maximum OT production of 0.92 g/L was achieved at low dilution rate (D=0.25 day^{-1}). The results demonstrate that optimal OT production with alginate immobilized cells could be achieved at a dilution rate of 0.25 day^{-1} in FBR.

Discussions

A three-phase fluidized bed reactor was used for continuous production of OT by immobilized cells *S. varsoveinsis* cells. Continuous reactor was operated successfully for

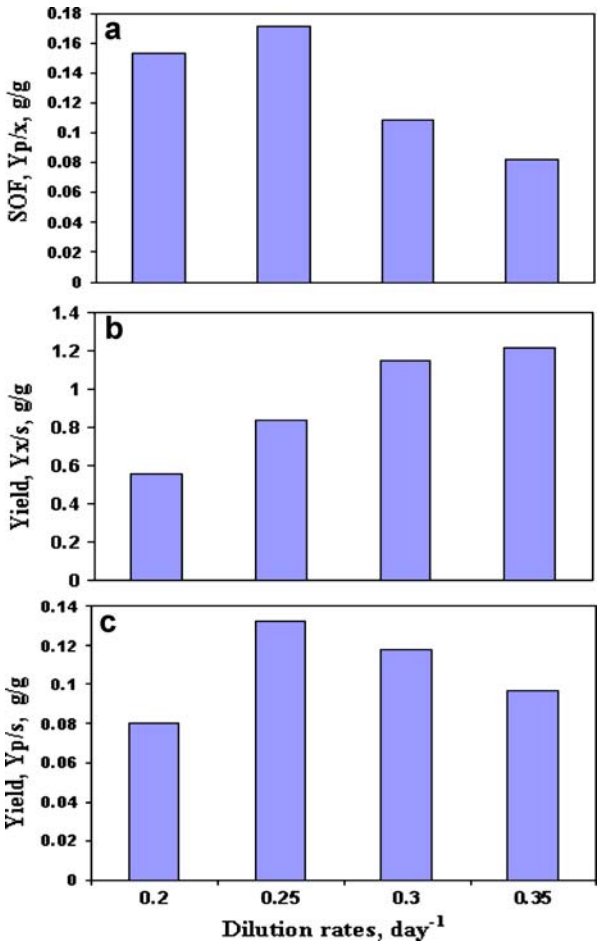


Fig. 5 Yield kinetics of OT in FBR under different dilution rates. **a** Specific OT formation (*SOF*), $Y_{P/X}$; **b** Biomass yield with substrate, $Y_{X/S}$; **c** Product yield with substrate, $Y_{P/S}$

21 days with three different concentrations of glucose and residence times. The feed concentrations were carefully manipulated to ensure maximum productivity and at the same time preventing the outflow of unutilized carbohydrates to the downstream. Three different feeding strategies were studied at 10, 15, and 20 g/L of glucose concentrations. Feeding of glucose at 20 g/L decreased OT production to 0.47 g/L from 0.75 g/L at 10 g/L of glucose. This is probably due to catabolic repression exerted by high concentrations of glucose source at 20 g/L [17]. The results showed that optimum OT production with alginate immobilized cells was achieved at a dilution rate of 0.25 day^{-1} in the FBR with 32% utilization of sugars.

Earlier workers [7] with *S. rimosus* immobilized on polyrethane have studied in a 220 mL air mixed reactor with 6 mL/h flow rate for oxytetracycline production in continuous mode of operation in stirred tank reactor with air sparging. However, Ca-alginate immobilization with glutaraldehyde as cross-linking agent used to prepare *S. varsoviensis* beads were used for OT production in a fluidized bed reactor. Our study shows that increase in dilution rate increases $Y_{X/S}$ and consequently results in the reduction of OT production. Since OT is a secondary metabolite, its production enhances at lower growth rates. The overall productivity of OT was improved by three to four times compared to that encountered with conventional batch fermentation. The steady decline in the antibiotic production from 0.627 to 0.312 g/L in FBR suggests that the cells were slowly being inactivated by aging brought about by the deficiency of nutrients in the medium.

The FBR was resulted in higher yields of OT because it provides better mass transfer rates than the STR. Control of operating parameters are easier in FBR than in STR, and also, the shear stress on immobilized cells in FBR is less when compared with STR [2, 3, 13]. Feeding of high glucose concentration (20 g/L) decreased OT production to 0.47 g/L. This is probably due to catabolite repression exerted by high concentration of the carbon source [17]. The majority of continuous culture studies for antibiotic fermentations have found out that the optimum specific production rate was obtained at low dilution rates, e.g., the studies of Lee [18] on tylosin production by *Streptomyces fradiae* NRRL 2702, Morikawa et al. [19] on bacitracin by immobilized living whole cells of *Bacillus* sp., and Adinarayana et al. on neomycin production by *S. marinensis* NUV-5 [20]. The carbohydrate consumption are in line with present FBR studies, and these results are also in line with an earlier work carried out for cephalosporin C production by bioparticles of immobilized cells of *Cephalosporium acremonium* ATCC 48272 in a repeated batch tower bioreactor as an alternative to the conventional process [21]. Rapid consumption of glucose was observed in subsequent batches of operation after the first batch of operation. Carbon dioxide produced during microbial metabolism could be effectively removed using column reactors for secondary metabolite production [22].

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