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Olivanic Acid Production in Fed Batch Cultivation by *Streptomyces Olivaceus* MTCC 6820

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ABSTRACT

Olivanic acids, a compound of β lactum class have been studied due to their β-lactamases inhibiting nature. Effect of different medium components on olivanic acid (OA) production was evaluated by a soil isolated strain, Streptomyces olivaceus MTCC 6820 in batch cultivations with glycerol (1.5%) and soybean meal (2%) as a carbon and nitrogen source. Production of Olivanic acid was found to be maximum (50 mg/L) at the third day of batch fermentation. However the olivanic acid production was increased nearly eleven times (565 mg/L) with sustained production up to ten days in fed batch cultivations under glycerol limiting conditions. Present work investigate the best experimental conditions for the olivanic acid production in terms of selection of feeding material and feeding time in fed batch fermentations. Results suggest that low supplementation of glycerol (2%) provides significantly higher olivanic acid production in comparison to other examined conditions.

Key words: Olivanic acid, β-lactum, Streptomyces olivaceus, Fed-batch fermentation.

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INTRODUCTION

During our extensive screening program for the isolation of microbial cultures producing novel antimicrobial agents, an active strain of Streptomyces olivaceus MTCC 6820 was isolated from the soil samples collected from the hilly region of India [1]. S. olivaceus is reported to produce diverse array of antibiotics such as elloramycin (polyketide), kanchanamycins (polyol macrolide), rabelomycin (anthraquinone) and beta-lactam ring containing carpetimycin or olivanic acids. The compound produced by the isolated strain was purified and chemically characterized as new form of olivanic acid. Olivanic acids are reported to inhibit β-lactamases [2, 3]. Olivanic acids and other β-lactamase inhibitors (clavulanic acid, tazobactam, and sulbactam) greatly enhance the therapeutic efficacy of their partner antibiotics such as amoxacillin, ampicillin, piperacillin etc. against common enteric and non-enteric organisms possessing beta-lactamases [4].

S. olivaceus was cultivated in a production medium containing glycerol as the carbon energy source. Olivanic acid, produced during the fermentation process was low in concentration. During fermentation, addition of suitable substrate to the production medium may enhance the yield of products. Fed-batch strategies have been considered as a means of controlling the growth rate, prolonging the stationary phase, overcoming substrate inhibition or metabolite repression and are used successfully in several studies [5, 6, 7]. Here to enhance the production of olivanic acids various experiments such as statistical optimization and fed batch experiments were performed. In the present work, effect of feed material and feed rate on the production of olivanic acid was investigated, using soybean meal and glycerol as a supplementary medium during fed batch experiments. One of the main advantages of the fed-batch process in relation to the simple batch process is the possibility of increasing antibiotic production by addition of substrates at a suitable flow rate [8].

MATERIALS AND METHODS

Producer organism- The producer organism, designated as AB, was isolated from a pretreated soil sample collected from hilly area of northern India and it was maintained on YMG slants containing (g/L) yeast extract 4.0, malt extract 10, glucose 4.0, $CaCO_3$ 2.0 and agar powder 20. Strain was taxonomically characterized on the basis of 16S rRNA homology and has been submitted at the Microbial Type Culture Collection (MTCC), IMTECH Chandigarh, India as S. olivaceus MTCC 6820.

Cultivation conditions and fed batch protocols- Three sets of fed-batch (FB1-FB3) and batch (B1-B3) experiments were performed in 1L Erlenmeyer flasks in a production medium (soybean meal 10 g, $CaCO_3$ 3 g, $MgSO_4.7H_2O$ 0.5 g, $(NH_4)_2HPO_4$ 0.5 g, NaCl 3 g, K_2HPO_4 1 g, glycerol 15 ml, DW 1L, pH 6.9-7.0) at 28^oC, 200 rpm to compare the production of olivanic acid. Samples were taken regularly at time intervals of 24 h and olivanic acid production was studied by assaying antimicrobial activity against B. subtilis ATCC 6633 by agar well method and finally on HPLC.

Packed cell weight was measured in triplicate by centrifugation of 10 ml of broth at 11086 g for 20 min followed by washing with demineralized water and again centrifugation for 20 min.

In the first set of fed batch experiment (FB1A-D) influence of feeding time was examined through single pulse feeding of 10 % (v/v) of production media at different time period i.e. 24, 48, 72 and 96 h respectively and the results were compared with the control (B1).

In second set of fed batch experiment (FB2A-C), influence of individual medium components such as glycerol, galactose (as a substitute of glycerol) and soybean meal on the production of olivanic acid was examined by feeding these medium components separately in definite concentrations at particular time which was found significant from the first set of experiments.

In the third set of fed batch experiment (FB3A-D), olivanic acid production was studied in the supernatant of samples taken during a fed-batch experiment. Glycerol concentration was determined by HPLC. During HPLC, NaOH (1 mM) solution was used as the mobile phase with a flow rate of 1 ml/min. The values for volumetric productivity of the olivanic acid for batch and fed-batch cultivations were calculated with eq. 1

$$C_{OA} = q/t \qquad (1)$$

Where q is the volumetric productivity of olivanic acid (mg/L/h), t is the cultivation time (h), C_{OA} is the olivanic acid concentration (mg/L).

RESULTS AND DISCUSSION

Isolated strain Streptomyces olivaceus (Fig. 1) was found to produce olivanic acid. Olivanic acid concentration in the production medium was 50 mg/L and it was enhanced nearly eight times (415 mg/L) by using statistical designs in batch cultivations [9]. For further enhancement in the production of olivanic acid other alternatives such as fed-batch process was used which is a well established process than the batch process for the production of β lactum antibiotics [8].

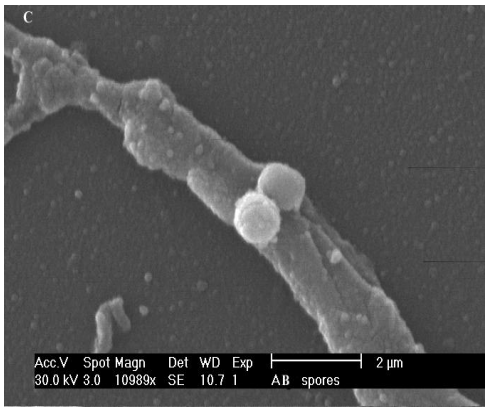

Fig.1 Electron microscopy of S. olivaceus showing filament and spore

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Effect of feeding time on olivanic acid production- S. olivaceus was cultured in a complex medium with a glycerol-soybean ratio of 3:4. Batch fermentations with this medium were characterized by a medium to high initial volumetric productivity of olivanic acid (qolivanic acid, 4.9 mg/L/h) upto two days and decreases rapidly as the fermentation progressed after 72 h. For fed batch fermentation conditions, feeding time had a considerable effect on the quantitative antibiotic production in relation to the cultivation period. It was observed that feeding of production medium when initiated during initial fermentation period i.e. the period of high qolivanic acid, results some increase in olivanic acid production (Fig. 2A). However this rate was decreased when the feeding was done at the later periods of growth (72 and 96 hr). Somewhat similar results were reported in the case of tylosin production by Streptomyces fradiae through fed-batch fermentations [10]. By using a cyclic feeding strategy, it is possible to increase olivanic acid productivity further.

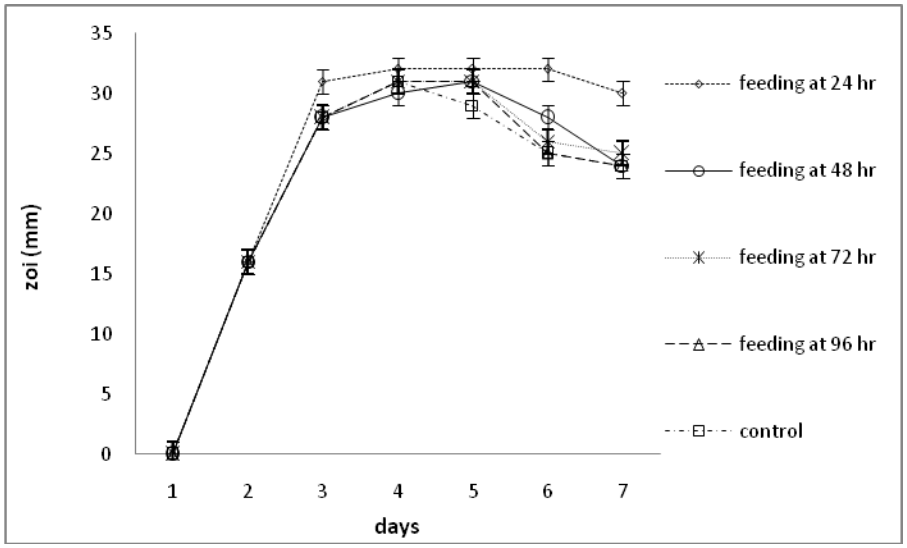

Fig.2 (A) Antibacterial activity in terms of zone of inhibition (zoi in mm) and (B) packed cell weight (PCW in g/l) of different fed batch experiments

Effect of variation in feeding material- The competitive position and potential profits from fermentation products are closely related with the cost of medium components [11]. In this context, Fed-batch experiments (FB2A-C) were started with the same batch medium containing glycerol, soybean meal and salts. Different feeding materials (2 % glycerol, galactose or X-medium) were fed at a constant rate at regular time interval and results were compared with the control.

It was observed that the experiments where glycerol, galactose or X medium were used as feeds, a sustained and enhanced antibiotic production was obtained in comparison to control (without feeding). By cyclic period of the substrate feeding, it was found that maximum olivanic acid accumulated (565 mg/L) when the glycerol cycle amplitude was 20 mg/L per cycle period of 24 h. Under optimum cyclic conditions it was possible to maintain maximum olivanic acid concentration and a constant value of qolivanic acid up to 240 h (Fig.3). Glycerol is the

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cheaper feeding material in comparison to galactose or complete production medium and as it provides better results it can be used as feeding component in the production of olivanic acids.

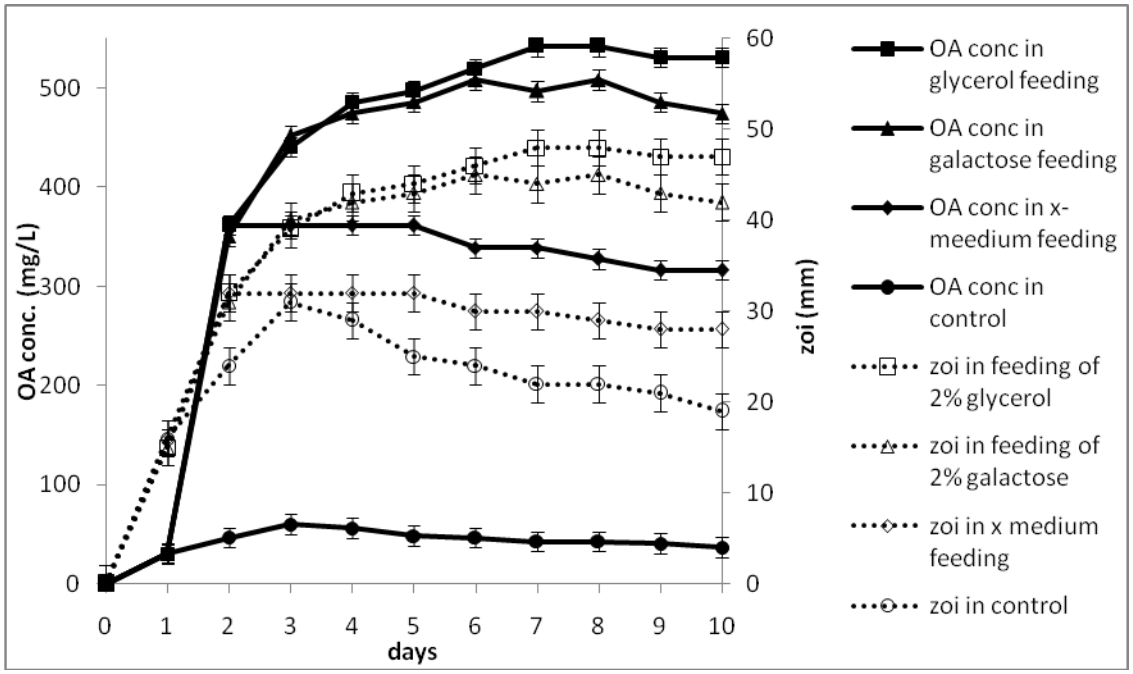

Fig.3 Antibacterial activity (zoi in mm) against B. subtilis ATCC 6633 and olivanic acid (OA) concentrations (mg/l) in batch (control) and fed batch experiments

Study of olivanic acid production- A comparison of the maximum value of concentration of (C_{max}) and the maximum value of volumetric productivity of olivanic acid (q_{max}) obtained in the two modes of operation is given in Table 1. Results suggest that the highest maximum concentration and the highest volumetric productivity of olivanic acid were obtained in the fed-batch cultivation. Maximum concentration of olivanic acid obtained in the fed-batch cultivation is a typical result; since this mode of operation favors production of secondary metabolites such as clavulanic acid, which occurs mostly during the stationary phase [12]. Glycerol feed increased the tenure of stationary phase, characterized by high cell concentration and an increased and steady rate of secondary metabolite production.

Table 1: Maximum concentration and productivity of olivanic acid obtained from batch and fed- batch experiments

Experiment	Olivanic acid conc. ($mg.l^{-1}$)	Maximum volumetric productivity ($mg.l^{-1}.h^{-1}$)
Batch	50	0.65
Fed batch	565	2.35

In the batch cultivation, a decrease in the rate of olivanic acid production was observed after 72 hours. At this time the cell concentration was still high and the glycerol concentration was lower than the value that causes repression or inhibition of olivanic acid production. This indicates that, in this process, the decrease in production rate is associated with the

biosynthesis of metabolic byproducts that repress or inhibit the metabolic pathway to olivanic acid. Fed batch cultivation somehow decreases the production of byproducts which may be responsible for the degradation of olivanic acid or inhibition of olivanic acid production.

CONCLUSION

The methods and results presented in this work demonstrate the feasibility of performing fed-batch production in shake flask level. The results for fed-batch culture indicate significant enhancement (nearly eleven times) in the olivanic acid concentration in comparison to batch cultivation. This suggests a significant reduction in medium requirements and experimental costs compared to batch production.

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