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Effect of Natural Inorganic Complex Material (Vermiculite) on the Production of Oxytetracycline by *Streptomyces rimosus*

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Abstract

In the present investigation, the addition of vermiculite, an inorganic natural complex material, to the cultivation medium resulted in a significant increase in both specific and volumetric oxytetracycline productions by *Streptomyces rimosus*. The addition of vermiculite at 30 g l^{-1} increased the total volumetric production of antibiotic by about 125%. On studying the optimal time for the addition of vermiculite to the cultivation medium, we observed that the addition of this material prior to cell cultivation (t_0) showed the highest effect on antibiotic production. However, the supplementation of vermiculite to the medium did not show any significant effect on other cultivation parameters such as optimal pH and antibiotic production time.

Key words: Oxytetracycline, vermiculite, *Streptomyces rimosus*

Introduction

The production of oxytetracycline (OTC) is generally regulated by different medium components such as carbon and nitrogen sources (Orlova, 1971; Abou-Zeid *et al.*, 1977; Abou-Zeid *et al.*, 1981; Bobyleva and Vorlova, 1985; Abou-Zeid *et al.*, 1993b; Rhodes, 1994;). Other medium ingredients such as phosphorous is also important for the growth of streptomycetes and regulation of OTC biosynthesis (Martin, 1997). Excess concentration of phosphorous (more then 6.0 mg%) causes, the mycelium propagates intensely, the consumption of carbohydrates increase, the pH of medium decrease and consequently the biosynthesis of OTC reduces. Beside these main medium ingredients, trace elements may also play a critical role in the production of many antibiotics. Microelements may act as structural elements or be part of enzymatic cell systems. Egorov (1985) found that the effect of iron on OTC production depends on the concentration of iron, the composition of the medium and the productive strain. Also, the production of antibiotics such as actinomycin, neomycin, candicidin, streptomycin and mitomycin requires manganese in the cultivation medium (Weinberg, 1970). Magnesium is also required for the production of leocomycin and spiramycin and the addition of magnesium sulphate to the cultivation medium increases the antibiotic concentration hundred fold (Higashide, 1984).

On the other hand, addition of other elements to the fermentation medium such as magnesium, sodium and other salts may be used for buffering purpose. However, these microelements may de added in pure form or via the addition of natural products containing these elements to the fermentation medium like molasses or date products (Abou-Zeid *et al.*, 1991; Abou-Zeid *et al.*,

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1993a; Abou-Zeid *et al.*, 1993b). These natural products contain enough microelements to satisfy any requirement for cell growth and OTC production. In the present work, the dehydrated magnesium-aluminum-iron silicate which is known as vermiculite was used as the medium formulation. This material contains sufficient quantities of trace elements such as (Mg, Al, Fe, K, Ca, Cr and Mn) which are necessary for OTC production. Vermiculite has many biotechnological applications as a good support for both of enzyme and cell immobilization (Chellapandian and Sastry, 1992; Anita *et al.*, 1992). Also, the addition of vermiculite to the cultivation medium as a source of nutrient for cell mass production was studied by Graham-Weiss *et al.* (1987). They showed that the direct fermentation of nutrients supplemented vermiculite offers a reliable process for production of bacterial inoculants. The integration of vermiculite in the culture enhances the production of bacterial inoculants for different strains of *Rhizobium*, *Pseudomonas* sp. and *Bacillus* sp. The report on the effect of vermiculite on the production of antibiotics in this paper has the priority.

Materials and Methods

Microorganism and inoculum preparation: Streptomyces rimosus (Pfizer 18234-2) was used in this study. The strain was maintained on the following medium (g l^{-1}): glucose, 10.0; peptone 5.0, KH_2PO_4, 1.0, $MgSO_4.7H_2O$, 0.5 and agar, 20.0. After 7 days of cultivation on this solid medium at 30°C, the arisen spores were harvested by gentle washing of agar surface with sterile saline solution. The inoculum size was 1×10^7 spore/ml.

Oxytetracycline production media and cultivation conditions: The simple OTC production medium (MI) contained the following ingredients (g l^{-1}): glucose 4.0, malt extract 10.0 and yeast extract 4.0. The pH was adjusted to 7.2 before sterilization. This medium was used either as such or after addition of different concentrations of the natural complex material, vermiculite (MII). Vermiculite was dried in an oven at 100°C for 24 h. and added to the fermentation medium under aseptic conditions. The chemical composition of the third type of medium (M III) was similar to MI with the exception that the liquid used in medium preparation was a filtrate of vermiculite suspension (30 g l^{-1}) pre-shacked for 48 h on a rotary shaker at 200 rpm.

In all cases, cultivation was carried out in 250-ml Erlenmeyer flasks containing 50 ml of the liquid medium. Inoculum was in the form of spores (1×10^7 spores/ml) obtained from a densely sporulating culture grown on ISP-2 medium for 7 days as described before. The flasks were incubated at 30°C on a rotary shaker at 200 rpm for 96 h. Three flasks were taken at different time intervals for the determination of pH, cell dry weight and the produced OTC amount.

Determination of cell dry weight: In case of both of media (M I and M III), the cells in the cultivation medium were collected by centrifugation at 5,000 rpm for 10 min. and then dried at 110°C until constant weight. In case of M II, which contains some insoluble fractions due to the presence of vermiculite, the difference between the weight of inoculated and non inoculated medium was taken as the cell dry weight.

*Determination of oxytetracycline (OTC):*OTC was determined quantitatively according to the biological method of Abou-Zeid and Shehata (1969). A standard curve was drawn between the log of different concentrations of OTC and the inhibition zones of susceptible bacterium *Bacillus subtilis* NRRL B-543.

Results and Discussion

Influence of different concentrations of vermiculite on growth and OTC production

To evaluate the influence of vermiculite concentration on the growth of *S. rimosus* and OTC production, media with different concentrations (10-50 g l^{-1}) of vermiculite were used. It can be seen from figure 1 that vermiculite increased the OTC production in all applied concentrations. The optimal concentration of vermiculite was 30 g/l. The production of OTC reached 225 mg/l

(about 125% increase in antibiotic production compared to that of non vermiculite supplemented culture). The yield of antibiotic production based on the cell dry weight [$Y_{p/x}$] increased significantly in all vermiculite supplemented cultures compared to control medium (without vermiculite). Vermiculite supported the cell growth of *S. rimosus* with different extent and the maximal cell dry weight was obtained in cultures supplemented by 30 g l^{-1}. On the other hand, the value of [$Y_{p/x}$] was about 0.028 (g g^{-1}) in all vermiculite supplemented cultures. This indirectly indicates that the production of OTC in vermiculite supplemented culture is related with cell dry weight. The increase of antibiotic production in vermiculite supplemented culture may be due to the presence of some trace metals which enhance the antibiotic production via the increase of cell productivity.

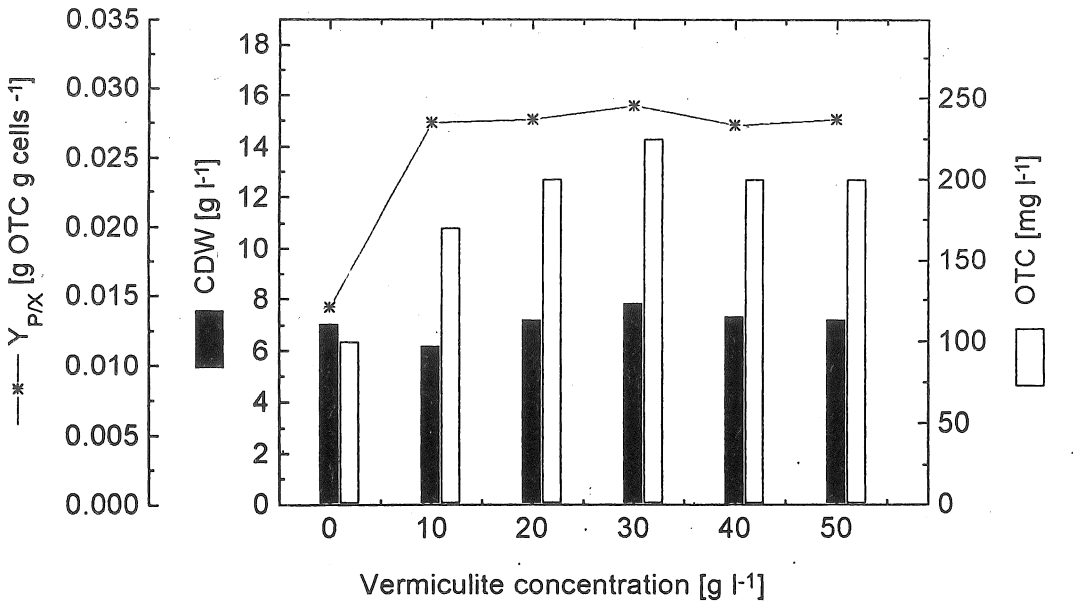

Figure 1: Effect of varying levels of vermiculite concentrations on the cell growth and oxytetracycline production by *Streptomyces rimosus*. (data were taken after 96 h cultivation)

Effect of vermiculite addition time on OTC production: In this experiment, the influence of addition of vermiculite at different periods of incubation on the production of OTC was investigated. The basal cultivation medium was supplemented with 30 g/l vermiculite at different incubation times. Table 1 shows that the maximal production of OTC (225 mg l^{-1}) was obtained when vermiculite was added at the beginning of the cultivation process (t_0). This value was about 125% higher when compared to the corresponding batch culture without vermiculite. If vermiculite was added after 24 h cultivation, the production of antibiotic increased by only 70%. On the other hand, the addition of vermiculite after 48 or 72 h cultivations showed no difference in OTC production compared to non vermiculite supplemented culture. The yield of antibiotic production [$Y_{p/x}$] reached 0.028 and 0.023 (g g^{-1}) in vermiculite supplemented culture at the beginning of cultivation time and for culture supplemented with vermiculite after 24 h, respectively. From these experiments, it is clear that vermiculite should be added at the beginning of the cultivation time. This may be due to the metals in vermiculite required to be added during the trophophase (cell growth phase) since if added after that time the effect of vermiculite will be negligible.

Table1: Effect of Vermiculite* addition time on growth and OTC production by *Streptomyces rimosus*.

Time of addition [h]	Final pH	CDW [g/l]	OTC [mg/l]	$Y_{[p/x]}$ [g OTC/g cells]
0	7.5	7.8	225	0.0288
24	7.4	7.6	170	0.0229
48	7.35	7.2	100	0.0138
72	7.35	7.2	100	0.0138
Control (without)	7.50	7.2	100	0.0138

*Vermiculte (30 g/L) was added at interval time and the control consists of fermentation medium MI, inoculated with *Str. rimosus* and incubated under the same cultivation conditions.

Kinetic of cell growth and OTC production in vermiculite supplemented cultures: Based on the results of the previous experiments, vermiculite (30 g l^{-1}) was added to the cultivation media at the beginning of cultivation time (t_0) to study its influence on S. rimosus growth and OTC production. For this purpose, three different media (M I, M II and M III) were used as described in materials and methods. The results in figure 2 show that the microbial growths in media M I and M III were similar, while M II yielded higher growth.

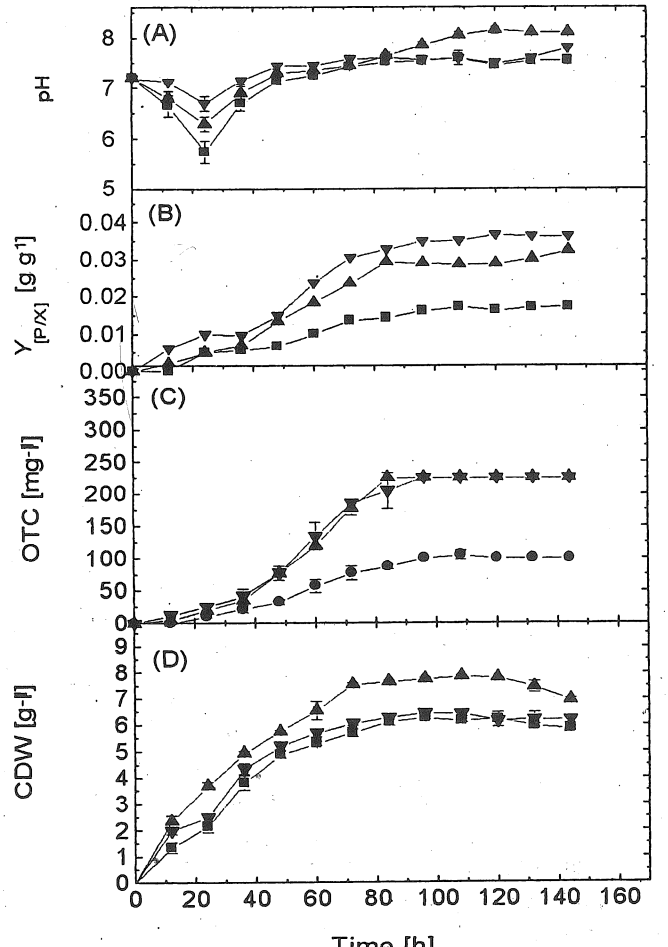

Figure2 : Growth cure for Oxytetracycline production and *Streptomyces rimosus* growth on different media. (■, medium I; ▲, medium II; ▼, medium III)

The production of OTC increased gradually with time and reached a maximal value of 225 mg/L in media M II and M III after 96 h. On the other hand, the maximal OTC production in M I (ISP medium) reached only 100 mg L^{-1} after that time.

The maximal yield of antibiotic production [$Y_{p/x}$] reached about 0.0294 and 0.036 (g g^{-1}) in media M II and M III, respectively. This indicated that, the enhanced antibiotic production in vermiculite supplemented cultures was due to the soluble fraction of vermiculite, whereas the insoluble fraction of vermiculite supports cell growth. The enhanced growth of some bacterial strains such as *Rhizobium*, *Pseudomonas* and *Bacillus* sp. due to the addition of vermiculite was also observed by Graham-Weiss *et al.* (1987). However, it is worthy to note that, the reduction in pH value during the growth phase for media M II and M III was less than in M I. This may due to the constituents of vermiculite which characterized by a good buffering capacity and prevent the drop in pH value.

Influence of the initial pH value on OTC production: The aim of this experiment was to investigate whether the addition of vermiculite to the cultivation medium changes the optimal pH of OTC production or not. Therefore, the effect of the pH on OTC production in different types of media was investigated. Figure 3 shows that the effect of pH on cell growth and OTC production in all cultures was almost the same.

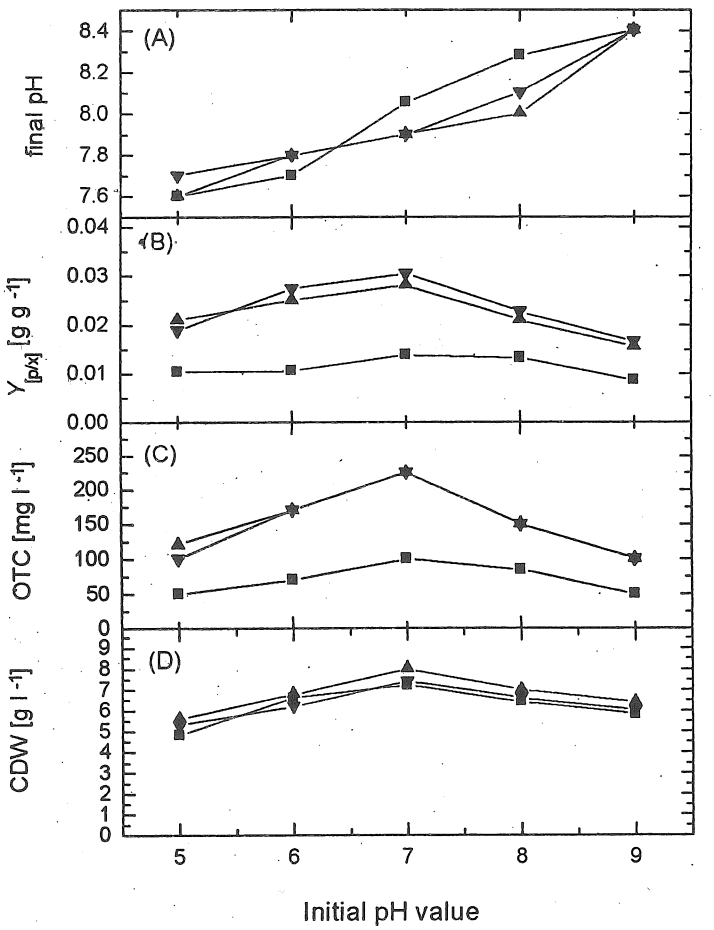

Figure 3: Effect of the initial pH value on the oxytetracycline production and cell growth by *Streptomyces rimosus*. (■, medium I; ▲, medium II; ▼, medium III).

The optimal pH for the biosynthesis of OTC was 7.0 in all media. Also, the values of volumetric antibiotic productions in medium II and III were almost the same at different pH values. For all applied pH values, the final pH lies between 7.4 and 8.4 depending on the initial value.

In conclusion, the integration of vermiculite, a natural inorganic material, to the cultivation medium is a cheaper alternative to other high cost pure inorganic salts and can be a suitable potential for the commercial production of oxytetracycline.

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