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Effect of support materials on antibiotic MSW2000 production by immobilized *Streptomyces violatus*

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The production of an antibiotic by free and immobilized cells of *Streptomyces violatus* through entrapment or adsorption on different materials was investigated. *S. violatus* entrapped in Ca-alginate beads gave low antibiotic activity compared to the free cell or adsorbed cell, while the adsorption of *S. violatus* on sponge cubes yielded the highest antibiotic concentration after 4 days of incubation in static cultures. A starch concentration of 10 g/L was optimum for the production of the antibiotic by adsorbed cells. The weight and size of the sponge cubes used for immobilization influenced production of the antibiotic and the optimum weight and size of the sponge were 0.8 g and 1.0 cm³, respectively, yielding a maximum antibiotic production of 280 µg/ml. Maximum antibiotic production was obtained at an initial pH value of 7.5 and in an inoculum size of 3 ml (spore suspension) per 50 ml. The production of the antibiotic in a fixed-bed bioreactor reached a maximum value after 2 days of incubation at a circulation rate of 30 ml/h. The immobilized cells in the bioreactor were reused seven successive times over a period of 14 days.

Key Words——antibiotic MSW2000; bed bioreactor; entrapment; solid support; *Streptomyces violatus*

Introduction

Immobilized cells have been defined as cells that are entrapped within or associated with an insoluble matrix. The application of immobilized cells to study microbial processes is one of the main trends in modern biotechnology. Immobilization techniques mostly employed for the production of secondary metabolites are cell entrapment and cell adsorption on solid supports (Asanza et al., 1997; Constantinides and Mehta, 1991; El-Sayed and Rehm, 1987; Roy et al., 1998; Webb et al., 1986). Cell immobilization shows many operational and economic advantages such as prolonged metabolic activities, reuse of the biocatalyst, in-

crease of cell concentration and preventing washing out of cells at higher flow rates (Cho et al., 1995; De Carvalho et al., 2000; Gautam et al., 2002; Koller et al., 2001; Kurilova et al., 2000; Wan et al., 1995).

In the last few years, the production of antibiotics and other secondary metabolites by immobilized microbial cells was regarded as a competitive alternative. Improvement of tetracycline production by *Streptomyces aureofaciens* cells immobilized in Ca-alginate, κ-carrageenan and within hollow fibers has been reported (Asanza et al., 1997; Kopp et al., 1984; Robertson and Kim, 1985). The production of erythromycin and neomycin by streptomyces cells entrapped in Ca-alginate and cellulose beads has also been studied (Bandyopadhyay et al., 1993; Park et al., 1994). Farid et al. (1994) and El-Enshasy et al. (1996) immobilized streptomyces cells onto glass wool or Ca-alginate and noted the effect of immobilization on oxytetracycline production. Sarra et al. (1997) immobilized *S. lividans*

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Fig. 1. Molecular structure of the antibiotic MSW2000.

cells by forming pellet aggregates of the organism and obtained high production of the antibiotic by using this preparation in a fluidized-bed reactor. El-Naggar et al. (1998) reported on the production of actinomycin X₂ in immobilized cultures of *S. nasri* YG62. However, the concern was directed toward the production of antibiotics by adsorbed microbial cells.

S. violatus used in the present investigation was found to produce only one major antibacterial substance (Hassan et al., 2001), and the structure of the antibiotic produced has been elucidated (El-Naggar et al., 2001). It was found to have a molecular formula of C₁₅H₂₃NO₃, a molecular weight of 276 and was designated as MSW2000 (Fig. 1). The aim of the present study was to verify the ability of immobilized *S. violatus* to produce the antibiotic under different experimental conditions.

Materials and Methods

Microorganism. *S. violatus* was isolated from garden soil, Faculty of Science (El-Shatby), Alexandria, Egypt. This strain was identified according to the International Streptomyces Project (ISP) scheme as described by Shirling and Gottlieb (1966) and the diagnostic key of Szabo (Szabo et al., 1975). It was maintained on nutrient agar slants and kept in a refrigerator at 4°C until further use. *Staphylococcus aureus* (209 P FDA) was used as a test organism for the antibiotic bioassay.

Culture media. Starch-nitrate was used as the basal medium for production of the antibiotic. This medium contained (g/L): Starch, 10.0; NaNO₃, 2.5; K₂HPO₄, 1.0; KH₂PO₄, 1.0; MgSO₄·7H₂O, 0.5; KCl, 0.5; and 1.0 ml trace salt solution containing (g/L): CuSO₄·5H₂O, 0.64; FeSO₄·7H₂O, 0.11; MnCl₂·4H₂O, 0.79; and ZnSO₄·7H₂O, 0.15. Medium was adjusted to pH 7.0 before autoclaving using 0.1 N NaOH or 0.1 N HCl solutions. Mueller-Hinton agar medium was used for antibiotic susceptibility testing and contained (g/L):

Beef infusion solid, 4.0; Starch, 1.5; Casein hydrolysate, 17.5; and Agar, 15.0.

Antibiotic bioassay. This was carried out by the paper disc diffusion method using Mueller-Hinton agar as the assay medium and *S. aureus* as the test organism (MIC of the purified antibiotic=0.38 µg/ml). The Mueller-Hinton agar (45°C) was poured into sterile Petri dishes (9-cm diameter) and allowed to solidify. A 0.1 ml bacterial suspension (3×10⁶ cells) of the test organism was inoculated into the agar surface. Sterile paper discs (6.0-mm diameter, Whatman antibiotic assay discs) were placed on the dried surface of the medium using alcohol-flame-sterilized forceps. Each disc received 20 µl of the culture filtrate. Petri dishes were kept in a refrigerator for 2 h to allow for the diffusion of the antibiotic. Petri dishes were then incubated inverted in a Köttermann incubator for 18–24 h at 37°C. The inhibition zone was measured in millimeters (Amade et al., 1994). The antibiotic concentration (µg/ml) was determined from a standard calibration curve using the purified antimicrobial substance (MSW2000) produced by *S. violatus*. Care was taken to confirm that no agonistic or antagonistic substances were produced during the production of the antibiotic in the bioreactor, and a HPLC assay was also carried out. The assay was performed on a Hewlett-Packard 1090A liquid chromatograph equipped with a scanning diode-array detector. The latter facilitated peak identification due to the characteristic UV chromophore (230 nm) of the antibiotic. The column used was a Spherisorb C18, 5 µm (25 cm by 4.6 mm) eluted with *n*-hexane:ethyl acetate (2:8 v/v) at a flow rate of 1 ml/min. Spectral scans were compared with the data obtained from the authentic samples of the purified antibiotic.

Immobilization and cultivation of *S. violatus*.

1. **Gel entrapment:** *S. violatus* spores were entrapped in 2% Ca-alginate gel beads (6×10⁶ spores/ml gel) as described by El-Naggar et al. (1998). Alginate beads (10 ml) were added to 50 ml of sterile starch-nitrate medium in a 250-ml Erlenmeyer flask. The flasks were incubated at 30°C on a rotary shaker (150 rpm) and in static cultures for 7 days. For free cultures, each 50 ml medium was inoculated with *S. violatus* spores equivalent to those used in the immobilized cultures. The antibiotic concentration was determined at different time intervals (2, 4, 5 and 7 days) and compared to those obtained from the free cultures.

2. **Adsorption:** Different solid supports obtained

from the local market (pumice particles, luffa pulp and synthetic sponge) and sintered glass beads were used. Sintered glass beads (3–5 mm sphere diameter and pore diameter of 60–300 μm) were purchased from Schött, Glasswerke, Mainz, Germany. The solid supports were in the form of particles (about 0.5-cm diameter) in the case of pumice, or cut to small cubic pieces (about 1.0-cm length) in the case of luffa pulp and synthetic sponge. Three milliliters of *S. violatus* spore suspension (2×10^6 spore/ml) were inoculated into each of the Erlenmeyer flasks (250 ml). Each flask contained 50 ml sterilized starch-nitrate culture medium and the solid support (about 0.6 g sponge cubes, 1.6 g luffa pulp, 4.0 g pumice or 4.2 g sintered glass beads). Nearly the same volume of the solid supports was obtained in all the flasks. The flasks were incubated in a rotary shaker at 120 rpm and in a static incubator (in Kötterman incubator) at 30°C for 4 days. Adsorbed *Streptomyces* cells were estimated as dry weight after washing the solid supports several times with distilled water, and then drying the adsorbed cells at 70°C. The dry weight of the adsorbed cells was determined by subtracting an average of the pre-determined dry weight of the support used from the weight of the support plus cells (El-Naggar et al., 1998). On the other hand, the culture filtrate was centrifuged and the antibiotic activity of the supernatant was determined against *S. aureus* as a test organism. Scanning-electron micrographs of adsorbed *S. violatus* mycelia on the solid supports used were produced using a Jeol JSM-5300 scanning electron microscope (JEOL Technics, Ltd., Tokyo, Japan) at the Electron Microscope Unit, Faculty of Science, Alexandria University, Egypt.

Antibiotic production in the fixed-bed bioreactor. The structure of the bioreactor used for antibiotic production is illustrated in Fig. 2. It was composed of a basal container (250-ml flask) containing 200 ml of sterile production medium inoculated with 15 ml of *S. violatus* suspension (2×10^6 spore/ml) and pumped into a glass column (4-cm inner diameter $\times$ 20-cm length) at a circulation rate of about 30 ml/min with the aid of a peristaltic pump. The column was filled with about 1.8 g of sponge cubes. The bioreactor was incubated in a Kötterman incubator at 30°C. For semi-continuous cultivation, the medium was removed after maximum production of the antibiotic was obtained (48 h), a fresh medium was added under aseptic conditions to begin a new run and the process was repeated several

Fig. 2. Schematic drawing of the fixed-bed bioreactor used for antibiotic production by adsorbed *S. violatus*.

times. Samples were collected from the flask at different time intervals for the estimation of antibiotic concentration, final pH and residual starch concentration (Dubois et al., 1956).

Results

Antibiotic production by free and immobilized *S. violatus*

The production of the antibiotic was investigated in free and immobilized cultures of *S. violatus* on different materials (Fig. 3, a and b). In shaken cultures, free cells show a better antibiotic production than immobilized cells in any of the tested materials. However, it was generally noticed that antibiotic production in the shaken cultures was lower than that obtained in static cultures in all cases. It is also shown that cultures containing cells entrapped in alginate beads yielded the lowest values of antibiotic concentration in static or shaken cultures. On the other hand, the adsorption of *S. violatus* cells on sponge cubes yielded the highest antibiotic production in static cultures, reaching 1.12-fold and 2.65-fold of that obtained by free and entrapped cells cultures, respectively. Comparative growth data on all adsorbents are presented in Table 1. The growth of cells in alginate beads as well as on

Fig. 3. Effect of different incubation periods on the production of an antibiotic by *S. violatus* cells immobilized on different materials under (a) static and (b) shaken conditions.

Table 1. Effect of different solid supports on the dry weight (mg/ml) of *S. violatus* after 7-day incubation in static and shaken cultures.

Solid support	Dry weight (mg/ml)	
	Static	Shaken
Luffa pulp	2.5	2.1
Sponge	4.0	2.3
Sintered glass	2.5	1.8
Pumice	1.6	1.8
Free cells	3.5	3.3

the surface of the tested solid supports was observed by scanning electron microscope (Figs. 4 and 5).

Fig. 4. Scanning electron micrographs showing the growth of *S. violatus* mycelia in alginate beads at (a) magnification of $\times 100$ and (b) magnification of $\times 500$.

Influence of starch concentrations on antibiotic production

Antibiotic production by adsorbed cells of *S. violatus* increased by augmenting the quantity of starch in the medium up to 10 g/L, where the highest value was recorded (180 µg/ml). The antibiotic concentration was 2.5-fold of that obtained at a starch concentration of 5 g/L. Starch concentrations higher than 10 g/L showed a gradual decrease in antibiotic production. It was also noticed that the dry weight of the cells increased by increasing starch up to 10 g/L, while higher or lower starch concentrations yielded a lower biomass.

Effect of weight and size of sponge cubes on antibiotic production

Utilization of a 0.2 g sponge cubes/50 ml medium for adsorption yielded the lowest antibiotic concentration

Fig. 5. Scanning electron micrographs demonstrating the adsorption of *S. violatus* mycelia on the pores and crevices of luffa pulp, sponge, sintered glass and pumice.

(72 µg/ml). A gradual increase of antibiotic production was obtained by increasing sponge cubes up to 0.8 g/50 ml medium, where a maximum value of 210 µg/ml was obtained showing an increase of 2.9-fold (Fig. 6). A further increase in sponge cubes showed an adverse effect on antibiotic production and a 44% decrease was noticed when 1.4 g/50 ml was used.

The size of the sponge cubes showed a similar effect on antibiotic production by immobilized *S. violatus* (Fig. 7). Maximum antibiotic production (280 µg/ml) was recorded in cultures containing sponge cubes of about 1.0 cm³, while the antibiotic concentration decrease by 24% and 40% in cultures containing cube sizes of 2.5 and 3.5 cm³, respectively.

Influence of inoculum size on antibiotic production

The results obtained indicate that by increasing the inoculum size up to 3 ml of spore suspension per 50 ml culture medium, the antibiotic concentration increased,

Fig. 6. Effect of different weights of sponge cubes on the production of an antibiotic by *S. violatus*.

Fig. 7. Effect of different sponge cube sizes on the production of an antibiotic by *S. violatus*.

reaching a maximum value of 245 $\mu\text{g/ml}$. This antibiotic concentration was about 5-fold of that obtained when a 0.5 ml/50 ml inoculum was used. Increasing the inoculum size above 3 ml/50 ml resulted in a gradual decrease in antibiotic production. Increasing the inoculum size also increased the dry weight; the highest dry weight values were obtained when using 3–5 ml/50 ml.

Influence of initial pH on antibiotic production

An initial pH of 7.5 favored the highest antibiotic production (280 $\mu\text{g/ml}$) and the antibiotic concentration was about 1.1-fold of that obtained at pH 7 (Fig. 8). Likewise, maximal dry weight (4.2 mg/ml) was obtained when the fermentation medium was originally adjusted to the initial pH 7.5. An initial pH value below or above pH 7.5 had a lesser promoting effect on production of the antibiotic as well as on growth. Initial pH values of 5.5 or 9.5 caused a decrease in the antibiotic concentration, showing only 14% and 18% of the values obtained at initial pH 7.5, respectively.

Production of antibiotic in a fixed-bed bioreactor

Antibiotic production by adsorbed *S. violatus* in a fixed-bed bioreactor was investigated. The data shown in Fig. 9 indicate that a relatively high antibiotic concentration was obtained after the first day of incubation (107 $\mu\text{g/ml}$) and increased to reach a maximum value (280 $\mu\text{g/ml}$) on the second day of incubation. It was noticed that, at longer incubation periods, the antibiotic concentration decreased gradually until the seventh

Fig. 8. Effect of initial pH of the fermentation medium on the production of an antibiotic by *S. violatus* cells adsorbed on different sponge cubes.

Fig. 9. Effect of different incubation periods on the production of an antibiotic by *S. violatus* in a fluidized-bed bioreactor.

day of incubation, where the culture showed an antibiotic concentration of 107 $\mu\text{g/ml}$, representing 38% of the antibiotic concentration obtained on the second day of incubation.

Effect of different circulation rates on antibiotic production

From the data obtained, it was shown that when the circulation rate was slow (10 ml/min) the antibiotic appeared in the culture after 24 h of fermentation, reaching 40 $\mu\text{g/ml}$ and a residual starch concentration of

37.7%. The antibiotic concentration increased gradually and reached maximum value (280 µg/ml) after 96 h, where starch was nearly consumed from the culture medium. When using a circulation rate of 30 ml/min, the suitable time for maximal antibiotic production was shorter. In that case, the antibiotic activity increased regularly during the first 24 h. The antibiotic concentration reached a value of 92 µg/ml, while a maximum antibiotic concentration (280 µg/ml) was reached at 48 h of incubation and the residual starch concentration was about 1.8%. However, when the circulation rate was rapid, about 65 ml/min, no antibiotic production was observed in the first 48-h incubation period and there was a high quantity of starch in the culture medium. Antibiotic production appeared at 58 h of incubation, reached only 40 µg/ml and remained relatively low until the end of incubation (96 h).

Effect of reusing adsorbed S. violatus cells in a fixed-bed bioreactor

The results indicated that the reuse of *S. violatus* adsorbed on the sponge yielded antibiotic production in the fermentation medium after each reuse for seven successive times over a period of 14 days. The highest antibiotic concentrations were recorded in the first and second runs, and then the antibiotic concentration decreased until the seventh run, which showed the lowest antibiotic concentration (40 µg/ml).

Discussion

The present research deals with the study of antibiotic production by a *Streptomyces* strain. The study included the utilization of different cell immobilization techniques and their effect on the production of an antibiotic.

The results showed that the entrapment of *S. violatus* in Ca-alginate beads yields a relatively low amount of antibiotic, where oxygen diffusion through the beads may affect the metabolic activity of the entrapped *S. violatus*. The porosity of the gel preparation employed, as well as the size of the diffusing molecules, may also affect the recovery of the antibiotic in the culture (Stormo and Crawford, 1992).

These results collectively indicate that the entrapment of *S. violatus* in Ca-alginate is not reliable for antibiotic production. Accordingly, immobilization by adsorption on different solid porous supports was taken into consideration. Maximum antibiotic production was

recorded in cultures adsorbed on sponge as a solid support. It was noticed that cultivation of sponge-adsorbed *S. violatus* in static cultures increased the production of the antibiotic, reaching 2.9-fold of that obtained in shaken cultures. Sridevi and Sridhar (1999) and Devi and Sridhar (2000) reported similar results by observing high cephamycin C production by immobilized *S. clavuligerus* cells adsorbed on sponge as the best support material used for immobilization. The high production of antibiotic by adsorbed cells may be due to better contact between the mycelia of *S. violatus* and starch that were both adsorbed to extra- and intracellular spaces of the sponge (Groom et al., 1988; Proksch et al., 2002; Sutar et al., 1986). Moreover, the sponge-adsorbed cultures showed the lowest viscosity, which could allow a better mass transfer and oxygen mixing. Many investigators (Atkinson et al., 1979; Gautam et al., 2002; Kautola et al., 1990; Lee et al., 1998; Sutar et al., 1986; Vassilev et al., 1993) reported successful immobilization of filamentous microorganisms on such open porous supports as sponge, polyurethane foam, cotton bags, nylon scrubbers or polystyrene. The production of antibiotic by sponge-adsorbed *S. violatus* cells was strongly influenced by the quantity and size of the solid support (sponge cubes) used in the culture, as well as the size of the inoculum. Optimization of these parameters increased production of the antibiotic in immobilized cultures by 1.36-fold. It was observed that relatively larger quantities of sponge cubes (1.0 to 1.4 g/50 ml medium) resulted in a lower immobilized biomass and a lower antibiotic yield.

Maximum antibiotic production by sponge-adsorbed *S. violatus* in a fixed-bed bioreactor was obtained in a relatively short time (48 h), reducing the time needed to reach the same yield when using conventional static cultures. Fixed-bed bioreactors were also shown to be advantageous for the cultivation of immobilized microbial cells, enhancing cell activities for the production of several secondary metabolites (El-Aassar et al., 1988; Foster et al., 1983; Guo et al., 1990; Kiese et al., 1980). It was observed that the rate of circulation of the medium through the fixed biocatalyst particles had a pronounced influence on the productivity of the system. The optimum rate of circulation was 30 ml/min, yielding maximum antibiotic production in a relatively short time. At circulation rates higher or lower than 30 ml/min, antibiotic production by the system was relatively low, which may be a result of inadequate aera-

tion or nutrient availability necessary for the cell activities. Other investigators (Cho et al., 1995; Fazeli et al., 1995; Kang et al., 1998; Shioya et al., 1999) reported the importance of aeration and dilution rate for the production of antibiotics by *Streptomyces* species cultivated in a fermenter. The system was reused semi-continuously for seven successive runs. It is noteworthy to conclude that the present investigation showed some beneficial effects of immobilization, approving its suitability for the production of an antibiotic by *S. violatus*. Adsorption of the filamentous organism on porous sponge support proved the productivity of the cells and their stability when compared to conventional free-cell cultures.

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