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Full Length Research Paper

Repeated batch production of vancomycin using synthetic cotton fibers

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The production of vancomycin by free and immobilized cells of *Amycolatopsis orientalis* was investigated. The influence of immobilization through entrapment in Ca-alginate beads and adsorption on different materials including synthetic cotton fibers, glass wool, cotton cloth and absorbed cotton on the production was compared to free cells in shake flask cultures. Cells entrapped in Ca-alginate beads gave low antibiotic yield compared to free cells. Immobilization of *A. orientalis* on synthetic cotton fibers as well as on glass wool gave the highest vancomycin yield after 4 days of incubation and were the best support materials for cell immobilization and vancomycin yield was about two fold higher than that produced by freely suspended cells under the same cultivation conditions. The effect of medium volume with immobilized cells revealed that in case of 25 ml fermentation medium, the production of vancomycin was 50% higher than the use of 50 ml volume. The reuse of the immobilized cells on synthetic cotton fiber five times over a period of 20 days repeated batch fermentation was investigated. A slight decrease in vancomycin production was noticed in the three last cycles. In general, immobilization minimized the lag time for both cell growth and vancomycin production. These results suggest that immobilization by adsorption is promising for industrial application.

Key words: Vancomycin, immobilization, fermentation and *Amycolatopsis orientalis*.

INTRODUCTION

Vancomycin is one of glycopeptides antibiotics, inhibiting the synthesis of cell wall peptidoglycan of micro-organisms (Nagarajan, 1991). It exhibits a strong inhibition effect on Gram positive bacteria, such as coagulase-negative staphylococci (Gruer et al., 1984) penicillin-resistant strains of *Streptococcus pneumonia* (Goldstein et al.,1994); Gram-positive bacilli such as *Bacillus anthracis* and *Bacillus cereus* (Weber, 1988); Corynebacteria such as *Corynebacterium diphtheriae* and *C. jeikeium* (Jadeja, 1983); and many of the clinically important clostridial species such as *C. difficile*, *C. perfringens*, *C. botulinum*, and *C. septicum* that are sensitive to vancomycin (Watanakunakorn, 1984). Intraventricular application of vancomycin is an effective therapeutic regimen for the treatment of shunt-associated staphylococcal ventriculitis (Nagl et al., 1999).

Immobilized whole cells have been widely used in the production of industrially important chemicals as well as pharmaceutical important compounds. Generally, immobilization of cells could be carried out by either entrapment of the microorganisms in porous polymers or microcapsules or binding to an organic or inorganic support matrix (Klein and Ziehr, 1990). Adsorption in addition to its simplicity has the possible advantages of reducing or eliminating the mass transfer problems associated with polymer entrapped cells (Ogbonna, 1991). Different antibiotics have been produced by immobilized cells such as nikkomycin (Trück, 1990), oxytetracycline (Ogaki et al., 1986), penicillin (Keshavarz, 1990), rifamycins (Chung et al., 1987), cyclosporine (Chun and Agathos, 1991), and other antibiotics (Kundu et al., 1992; Yasouri and Foster, 1991). Many workers have studied the factors and chemical composition of the media favoring the fermentation production of vancomycin by free cells (Hyung-Moo et al., 2007).

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However, Padma et al. (2002) studied the optimum process conditions i.e. pH, temperature, inoculum size, agitation, and aeration for vancomycin production by *Amycolatopsis orientalis* ATCC 43491 and the response surface curves reached 686 µg /ml in 250 ml shake flask. To our knowledge, this is the first report on the vancomycin production by immobilized *A. orientalis* NRRL 2450 cells through entrapment and adsorption techniques.

MATERIALS AND METHODS

Microorganism

The vancomycin producing strain *A. orientalis* NRRL 2450 was provided by the ARS culture collection, Northern Regional Research Laboratory (NRRL), Peoria, Illinois, USA as well as the *Bacillus subtilis* NRRL B-543 used as the test organism for the antibiotic bioassay.

Culture media

A. orientalis NRRL 2450 producing strain was grown and maintained on ISP2 media slants consisted of the following (g/l): glucose,4 ; malt extract 10; yeast extract, 4 and agar 20. Flasks containing 50 ml ISP2 liquid media were inoculated with spore suspension to obtain 48 h growing vegetative inoculum for fermentation cultures. The basal medium for production of antibiotic consisted of the following (g/l): soybean (30); starch (20); $CaCO_3$ (10) and $MgSO_4.7H_2O$ (1), and was adjusted from pH 7.0 to 7.2. Muller – Hinton agar medium was used for antibiotic sensitivity test and it contained (g/l): beef infusion solid (4); starch (1.5); casein hydrolysate (17.5) and agar 20.

Immobilization and cultivation of *A. orientalis*

Free cells cultivation

3 ml suspension of 48 h growing vegetative cells was added to 50 ml of sterile fermentation medium in a 250 ml Erlenmeyer flask. The flasks were incubated at 30°C for 144 h. Evaluation of vancomycin yield was studied every 24 h. Cell dry weight was determined after filtration of the free cell cultures through Whatman 1, and was oven dried at 70°C over night until it reached a constant weight. The difference between filter papers before and after filtration represents the cell dry weight.

Gel entrapment

Centrifuged cells of 48 h growth of vegetative cells were added to 50 ml of 2% (w/v) sterile sodium alginate gel at 40°C with stirring. The obtained mixture was added dropwise to a cold sterile 2% calcium chloride solution under gentle stirring to form spherical beads particles as described by El-Naggar et al. (1998). Alginate beads equivalent to 3 ml suspension of 48 h growth vegetative cells were added to 50 ml of sterile fermentation medium in a 250-ml Erlenmeyer flask. The flasks were incubated at 30°C on a rotary shaker (180 rpm). The antibiotic concentration was determined each 24 h and compared to those obtained by the free cultures. The entrapped biomass was determined from the difference between the mycelium plus the supporting materials and the support materials itself.

Adsorption

Four supporting materials including; synthetic cotton fibers, absorbed cotton and cotton cloth were procured from the local market and glass wool was purchased from Coming Co., UK. 0.5 g of each supporting material was sterilized with 50 ml fermentation medium into each of the Erlenmeyer flasks (250 ml). 3 ml suspension of *A. orientalis* 48 h growth vegetative cells was inoculated. Upon studying medium volume effect, 1.5 ml suspension was inoculated in 25 ml fermentation medium including 0.25 g supporting material. Flasks were incubated in a rotary shaker at 30°C; immobilized biomass of *A. orientalis* was harvested from the medium and washed with distilled water. The dry weight of the biomass was determined as the difference between the weights of the oven-dried (70°C overnight) immobilized supporting material before and after microbial growth.

Repeated batch production of vancomycin from *A. orientalis* NRRL 2450 immobilized on synthetic cotton fiber was achieved by aseptic removal of fermentation medium, which was replaced with a fresh one. Vancomycin in the production medium was estimated at different time intervals each 24 h. Each replacement of production medium was designated as a reused and hence semi-continuous production.

Determination of vancomycin

Vancomycin was determined by the biological assay method according to Abou-Zeid and Shehata (1969). A biological standard curve was used between different concentrations of pure vancomycin [kindly provided by Egyptian International Pharmaceutical Industries Company (EIPICO)] and the inhibition zone diameter of the susceptible bacterium *B. subtilis* NRRL B-543.

RESULTS

Batch cultivation of free and immobilized cells

The data illustrated in Figure 1 show that the cultures containing cells entrapped in alginate beads yielded lower antibiotic value (0.34 g/l) compared with that produced by free cells during incubation times. On the other hand, the adsorption of *A. orientalis* cells on synthetic cotton fibers (Figures 2a and b) and glass wool yielded the highest antibiotic production (1.6 g/l and 1.5 g/l, respectively) followed by that obtained by immobilized cells on cotton cloth (1.1 g/l), absorbed cotton (0.8 g/l) as well as free cells which gave maximum production of 0.9 g/l. This observation revealed that the yield of production upon using synthetic cotton fibers and glass wool was twice higher than that obtained by the free cells cultures. It was noticed that the antibiotic production started earlier in free cell cultures than in immobilized ones by adsorption and increased by time until it reached its maximum value by 96 h (Figure 3). With further incubation, no improvement in antibiotic titer was observed. On the other hand, the maximum production period which lasts 144 h with free cells was increased to 192 h with immobilized cells. A comparative study was carried out to

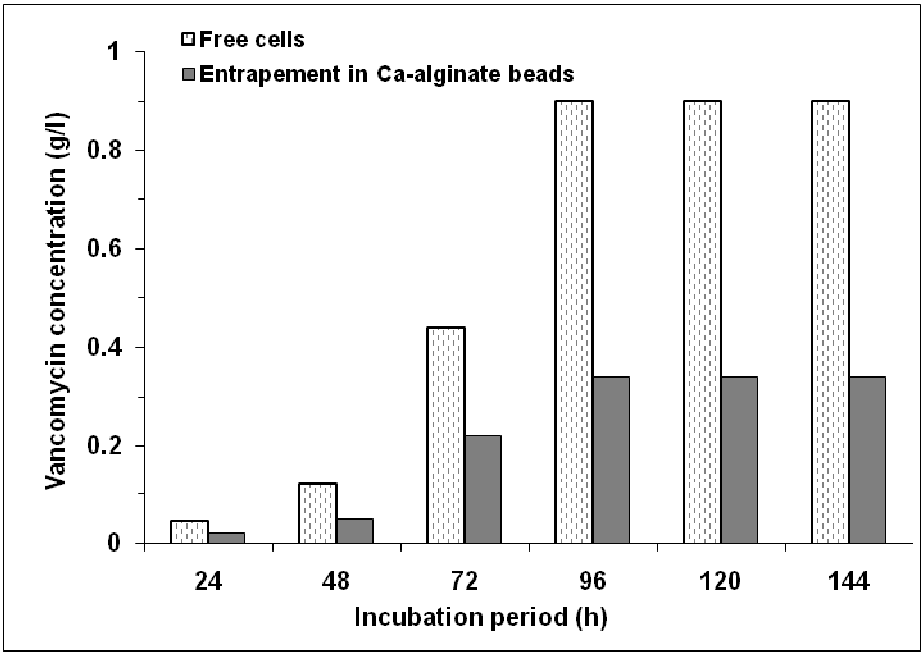

Figure 1. Effect of different incubation time on vancomycin production by *A. orientalis* in free and immobilized cells by entrapement in Ca- alginate beads.

investigate the cell growth, vancomycin production and specific activity of free and immobilized cells by adsorption as shown in Figure 4. The amount of cell biomass was varied from 2.8 to 4.0 g/l with the type of supporting material used. The specific antibiotic activity of free cells (0.3 g/g) was less as compared to that of immobilized cells on synthetic cotton fiber and glass wool that reached about 0.4 g/g and 0.37 g/g, respectively.

Repeated batch cultivation of free and immobilized cells

Vancomycin production by *A. orientalis* NRRL 2450 adsorbed on synthetic cotton fiber was studied up to five reused cycles as shown in Figure 5. During the first reuse, the vancomycin production increased gradually with the increase of fermentation time and reached a maximum of 1.6 g/l at the end of 96 h.

For the second reuse, the cells were already in the late exponential phase and hence a faster initiation of the vancomycin production was observed. Levels of vancomycin production were 1.6 g/l and 1.62 g/l at the end of 72 h and 96 h, respectively, as compared to 1.58 g/l and 1.6 g/l for similar time interval in the first reuse.

In the third reuse, a slight decrease in antibiotic production was reached (1.58 g/l) and (1.59 g/l) at the same time intervals. Further decrease in vancomycin production was detected in the fourth and fifth reuse until it reached its minimum value of 1.55 g/l at the end of 96 h. Free washed cells which yielded 0.9 g/l initially in the production medium showed a marked loss in the first reuse yielding only 0.8 g/l. Repeated batches of freely suspended cells was accompanied by a significant decrease in both cell growth and antibiotic titer. Production of vancomycin by *A. orientalis* NRRL 2450 in repeated batch cultivation upon using 25 ml medium volume was observed and represented in Figure 6. An enhancement of the production was attained and reached the maximum of 2.47 g/l after 72 h compared to 1.65 g/l in the second batch cultivation upon using 50 ml medium volume. On the other hand, repeated batch cultivation by the reuse of immobilized cells each 72 h compared to 96 h in the 50 ml medium volume was employed. Results obtained indicate that the immobilized cells on the synthetic cotton fibers used led to a significant stability in the antibiotic activity in comparison with free cells production and were able to maintain vancomycin at an almost constant high level making repeated batch production of vancomycin to be an attractive work.

DISCUSSION

The immobilization of *A. orientalis* NRRL 2450 cells by entrapment in calcium alginate beads gave lower yield of vancomycin than the free and adsorbed cells in batch cultivation. This result is in agreement with Farid et al. (1994b) who reported that the production of oxytetracycline by immobilized *Streptomyces rimosus* cells in calcium alginate gels was high. This may be due to diffusion limitation problems or nutrient deficiency which

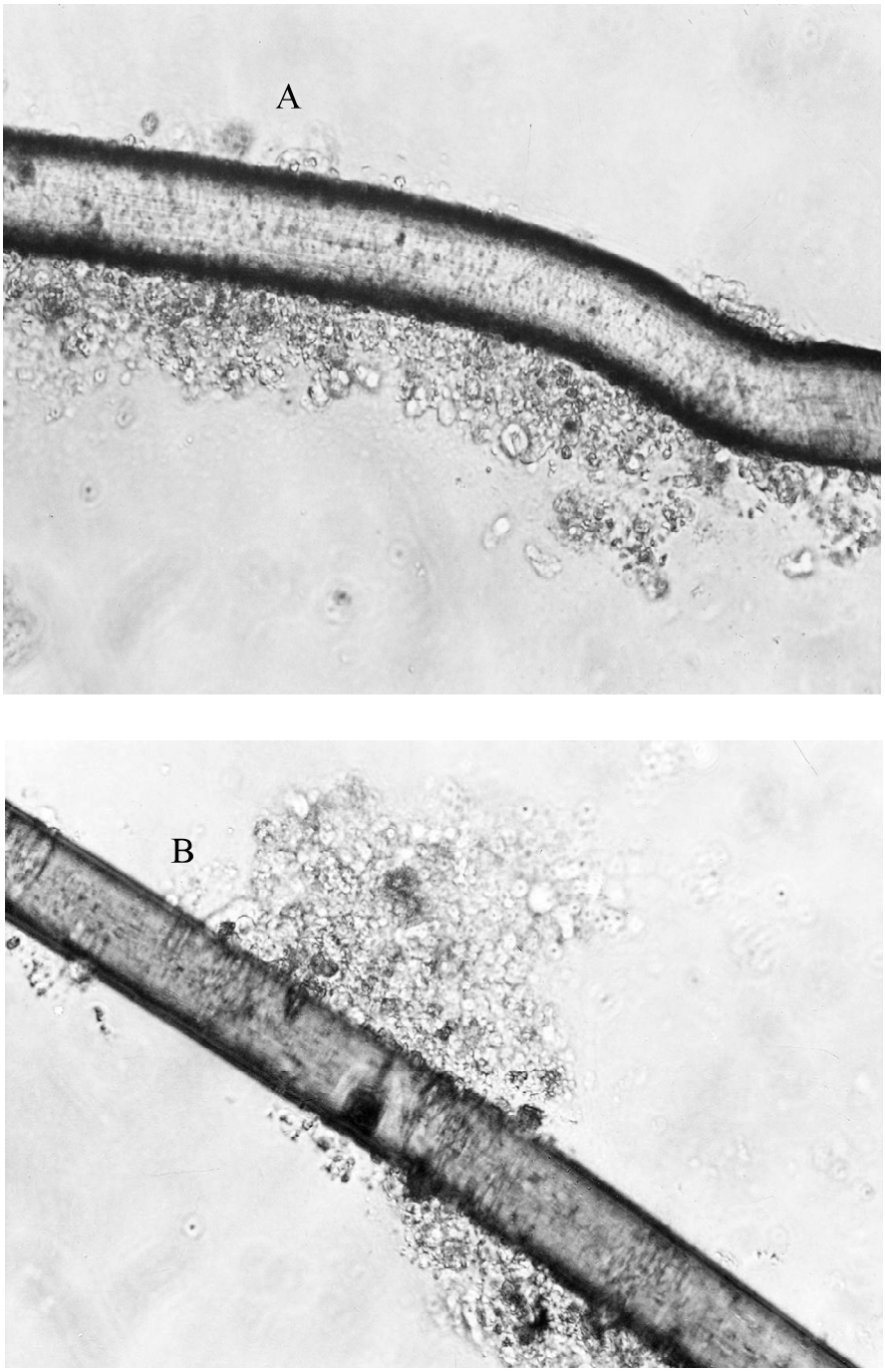

Figure 2. Microscopic observation of *A. orientalis* NRRL 2450 at early fermentation time; 24 to 48 h (Figure 2a) and 96 to 144 h (Figure 2b) showing spores on synthetic cotton fiber.

might be the reason for the lower production (El- Sayed and Rehm, 1987). Otherwise, the physical characteristics of the gel like, pore size and viscosity affect the diffusion of nutrients and oxygen as well as the biomass and production capacity as in the case of *Streptomyces aureofaciens* cells in which the formation of chlortetracycline is a strictly aerobic process (Hostalek et al., 1979).

A comparison of the vancomycin titer, cell mass production and specific activity with immobilized cells on various supporting matrices during 96 h fermentation cycle is shown in Figure 3. This variation of cell biomass may be due to the surface properties of supporting materials since the attachment of microorganism may

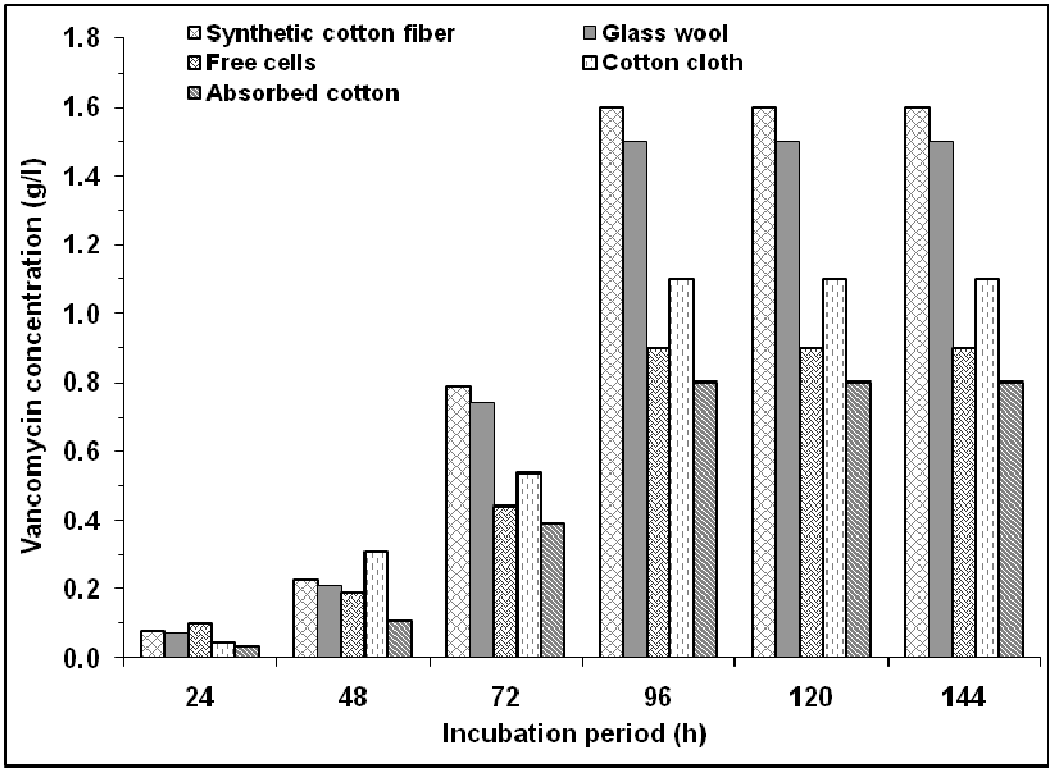

Figure 3. Effect of different incubation time on vancomycin production by *A. orientalis* in free and immobilized cells by adsorption on different support materials.

occur as a result of physicochemical interaction between the cell wall and the surface of the support material (Nelson, 1976; Lechevalier et al., 1986).

The use of synthetic cotton fibers to immobilize actinomycetes has significant effect. It is made up of interconnecting synthetic filaments with an open network of fibrous support giving the potential for rapid contact of immobilized cells with the surrounding aqueous medium. So the living cells adsorbed on it has very good diffusion of nutrient and oxygen transfer. Furthermore, the maximum percentage of cell mass adsorption onto synthetic cotton fibers, glass wool, cotton cloth and absorbent cotton may be due to the formation of network by the matrices. The specific antibiotic concentration with the immobilized culture was higher than that in the case of free cells. This can be explained by the stability of the intracellular biosynthetic factors and activities of the secondary metabolic enzymes of immobilized cells, as reported by Chibata and Tosa (1981) and Farid et al. (1994a, b). In addition, Atkinson et al. (1979), Gautam et al. (2002), Kautola et al. (1990), Lee et al. (1998) and Sutar et al. (1986) reported successful immobilization of filarnentous microorganisms on such open porous supports as sponge, polyurethane foam, cotton bags, nylon scrubbers or polystyrene. Moreover, the synthetic cotton fibers -adsorbed cultures showed the lowest viscosity, which could allow a better transfer and oxygen mixing. Hence, materials such as glass wool, synthetic cotton fibers were selected for repeated batch fermentation. Absorbent cotton and cotton cloths were not selected for further studies.

Repeated batch fermentation with immobilized cells on synthetic cotton fibers was carried out to evaluate the vancomycin production. Simple aseptic fermentation medium removal and replacement allows multiple reuses of cells in an economic way. It was observed that vancomycin productivity of the immobilized cells increased gradually during the early use cycles. This may be caused by the growth of cells inside the matrix as previously reported by Kokubu et al. (1981) and Abraham et al. (1991). The cells immobilized on synthetic cotton fibers continued to produce significant vancomycin titers for 20 days (5 batches) and a slight decrease in antibiotic titer in the last three cycles was observed, whereas during the incubation time of the cycles, cells immobilized were still able to produce the antibiotic. Repeated batch fermentations for vancomycin production were carried out with washed free cells; the loss in activity may be due to destruction of cells as well as autolysis which occurred during the centrifugation and washing process. When medium volume was changed from 50 ml to 25 ml, the production of vancomycin increased. According to the above investigation, it is essential to maintain enough supply of dissolved oxygen to improve the production of vancomycin. Further comparison of the increase of vancomycin concentrations in the broths suggested that

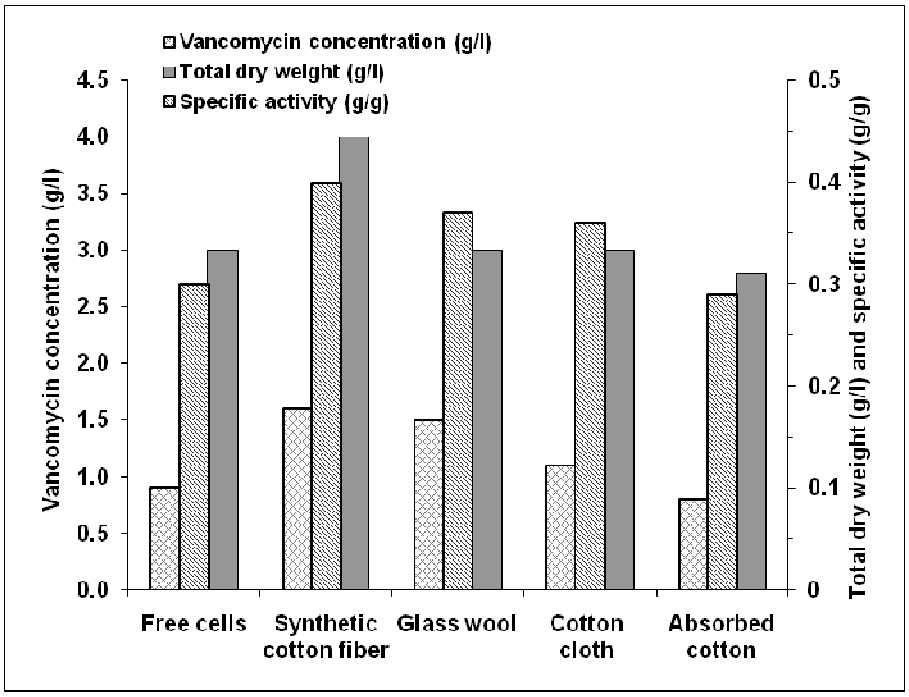

Figure 4. A comparative study between cell growth, vancomycin production and the specific activity on different support materials.

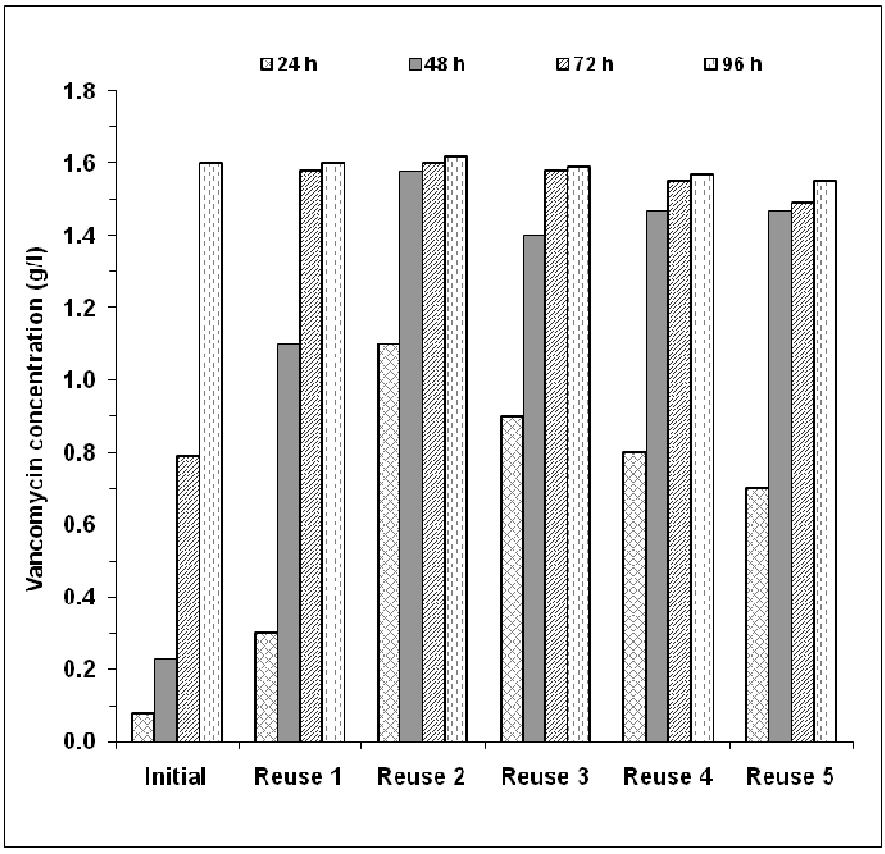

Figure 5. Production of vancomycin by *A. orientalis* NRRL 2450 in repeated batch cultivation upon using 50 ml medium volume.

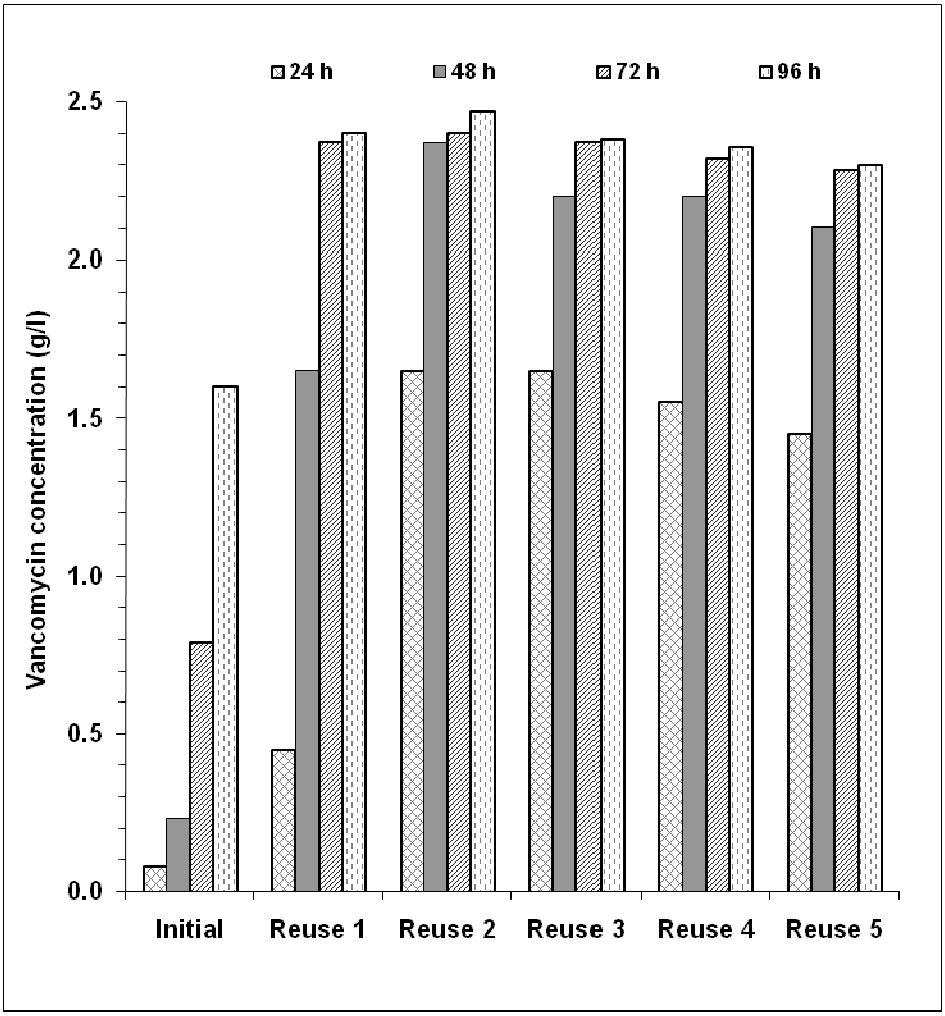

Figure 6. Production of vancomycin by *A. orientalis* NRRL 2450 in repeated batch cultivation upon using 25 ml medium volume.

the influence of shearing force was less serious than that of dissolved oxygen during the fermentation process. Compared to the original production of vancomycin (1.65 g/l) upon using 50 ml medium with this immobilized strain, an improvement of vancomycin production was achieved which reached 2.47 g/l by using 25 ml medium in the shake flask scale.

From the data, it is clear that immobilized cells of *A. orientalis* NRRL 2450 could be reuse for the production of vancomycin in repeated batch fermentation in shake flask process. Similar observations were reported by Farid et al. (1994 a) where the immobilized growing cells on glass wool were reused and continued to produce oxytetracycline by *S. rimosus* and rifamycin B and SV by *Amycolaptosis* for 20 days. Similarly, investigators studied on cells immobilized by adsorption technique on various supporting matrices for different antibiotics production and reported the antibiotic yields are high compared to free cell fermentations (Gekas, 1986; Karhoot et al., 1987; Joshi and Yamazaki, 1987; Chetsumon et al., 1993).

Among various supporting matrices, glass wool and synthetic cotton fibers were found to be good support materials, while absorbent cotton and cotton cloth were found to be poor for whole cell immobilization as well as antibiotic production. Although, cotton cloth and absorbent cotton are good materials, they are compressed in comparison with the synthetic cotton fibers which are loose fibers which permit oxygen diffusion. In addition to the advantage of the synthetic cotton fibers as support material for immobilization in repeated batch fermentation for 20 days, it was stable under sterilization conditions and low cost raw material.

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