Rifamycins Production by *Amycolatopsis mediterranei* in Batch and Repeated Batch Cultures Using Immobilized Cells in Alginate and Modified Alginate Beads

1Hesham A. El-Enshasy, 2Usama I. Beshay, 2Ahmed I. El-Diwany, 2Hoda M. Omar, 2Abdel Ghany E. El-Kholy and 2Rabab El-Najjar

1Department of Bioprocess Development, Mubarak City for Scientific Research and Technology Applications, New Burg Al-Arab, Alexandria, Egypt  
2National Research Centre, Tahrir Street, Dokki, Cairo, Egypt

**Abstract:** The production of rifamycins B and SV by *Amycolatopsis mediterranei* using immobilization technique was investigated. Alginate beads proved to be an alternative method for the production of rifamycin antibiotic as it has many advantage over using free cells such as it enables the operation at higher dilution rates without the danger of wash out, ease of handling and facilitates recycling or reverse of using microorganism. Different hardening agents were used to improve the stability of the beads and decrease cells escape. Gum Arabic and gellan gum were used and the rate of rifamycin production increased by using gellan gum as hardening agent compared to pure alginate beads. The rifamycins B and SV increased from 685 to 810 and from 547.5 to 702 mg L\(^{-1}\), respectively by using gellan-gum modified beads compared to the non-treated beads. In repeated batch production, the beads were stable and no breaking of the gel beads was observed throughout the experiment time. The total amount of rifamycin B and SV during the five repeated batches (each of 144 h) reached 3360 and 2309 mg L\(^{-1}\), respectively. On reducing the batch time to only 72 h, the antibiotic production was not influenced and the total concentration of rifamycins B and SV for five batches was 3785 and 2393 mg L\(^{-1}\), respectively.

**Key words:** Rifamycins, *A. mediterranei*, cell immobilization, repeated batch cultivation

**INTRODUCTION**

The Rifamycins are a family of ansamycin antibiotics produced by *Amycolatopsis mediterranei* antibiotic. Of different forms of rifamycins, rifamycin B, S and SV and the most active type. These forms are highly active against gram-positive bacteria (G+ve) and Mycobacterium tuberculosis and has a limited activity against gram-negative bacteria (G-ve). At present, reports on the clinical use of rifamycin have been generally confined to the treatment of tuberculosis, leprosy and AIDS-related mycobacterial infections (Sekpowitz *et al.*, 1995; Griffith and Wallace, 1997; Tripathi *et al.*, 2004; Lawn, 2005; O'Brien and Spigelman, 2005). Moreover, many derivatives of rifamycin B and SV such as rifampicin, rifapentine, rifaximin have wide applications in the treatment of enteric infections (Huang and DuPont, 2005; peptic ulcer (Ahuja *et al.*, 2005; Gasbarrini *et al.*, 2006), toxoplasma infection (Araujo *et al.*, 1996; Brun-Pascual *et al.*, 1998) and prevention of traveler's diarrhea (Sierra *et al.*, 2001; DuPont *et al.*, 2005). More recently, the rifamycin derivative (rifaximin) has been reported to be effective for the treatment of Hepatic Encephalopathy (HE) in Europe (Festi *et al.*, 2006; Shah and Kamath, 2006). This make rifamycins an attractive groups of antibiotics with growing market.

In spite of some few reports for the production of rifamycin in solid state fermentation (Venkateswarlu *et al.*, 2000), the industrial production of rifamycins is carried out mainly in submerged cultures using cells in free form. The main disadvantage of this process is long time cultivation and as typical secondary metabolite, the antibiotic production started when the cell entered stationary phase and continued during both stationary and decline phase.

**Corresponding Author:** Dr. Hesham A. El Enshasy, Department of Bioprocess Development, Genetic Engineering and Biotechnology Research Institute, Mubarak City for Scientific Research and Technology Applications, New Burg Al Arab, 21934 Alexandria, Egypt  Fax: +203 4593423

1381
Thus during traditional batch or fed-batch production of rifamycins by free cells, more than 50% of cultivation time are used for cell growth during the early time of cultivation (El Enshasy et al., 2003). On the other hand, large fractions of medium composition are used in cell growth in each batch. Thus the use of cells as retained biofactory inside the production vessel for continuous production is one of alternative production process. Therefore immobilized cell technology is considered, since its first application in early-1980s, as an alternative method for the continuous production of microbial cells for both primary and secondary metabolites. Different methods of cell immobilization and its possible applications were described in many books and reviews (Bierkens, 1997; Wijffels, 2001; Junter and Journee, 2004; Kourkoutas et al., 2004). The continuous production of antibiotics using immobilized cells were succeeded for the production of different types of antibiotics such as: Neomycin (Sririvasulu et al., 2002; Bandi et al., 2003), oxytetracycline (Farid et al., 1994; El Enshasy et al., 1996; El Enshasy, 2002), chlorotetracline (Tenuel et al., 1997), rifamycins (Chung et al., 1987; Abu-Shady et al., 1995; Farid et al., 1995), cephamycine (Devi and Sridhar, 2000), penicillin G (Mussenden et al., 1993) and many other antibiotics. The present work was undertaken to improve the production process of rifamycins by immobilized cells of *A. mediterranei* in alginate and modified alginate beads. Different parameters affecting cell productivity such as alginate concentration, bead size, cell loading inside alginate beads, age of inoculum, size of inoculum were studied. Moreover, the possibility for the reuse of immobilized cells in long term operation by means of repeated batch mode was also investigated.

**MATERIALS AND METHODS**

**Microorganisms:** *Amycolatopsis mediterranei* ATCC 21789 obtained from the American culture collection, USA. It was maintained on BENNET’s medium containing: (g L⁻¹): 10.0 glucose, 1.0 yeast extract, 1.0 beef extract, 2.0 N-Z amine, 20.0 agar. These ingredients were dissolved in 1 L of distilled water. The pH of the medium was adjusted to 6.8-7.0 before autoclaving. Agar slants were incubated at 28°C for 5-7 days.

**Inoculum preparation:** inoculum was prepared in vegetative culture medium containing (g L⁻¹): 10.0 Glucose, 3.0 KH₂PO₄, 1.5 K₂HPO₄, 1.0 MgSO₄·7H₂O, 0.016 FeSO₄·7H₂O, 0.001 Zn acetate, 5.0 yeast extract. The pH of the medium was adjusted to 7.0 with 1 M NaOH and the medium was distributed in flasks each containing 50 mL before autoclaving. Cultivations were carried out for 48 h at 30°C in incubatory shaker at 200 rpm (Infors Co., Switzerland). The obtained vegetative cells were used to inoculate shake flask in immobilization technique.

**Medium for rifamycin production:** Unless otherwise mentioned, the medium used in shake flask for immobilized cells cultivations was composed of (g L⁻¹): 40.0 Glucose, 3.0 KH₂PO₄, 1.5 K₂HPO₄, 1.0 MgSO₄·7H₂O, 0.016 FeSO₄·7H₂O, 0.001 Zn acetate, 5.0 yeast extract. The pH of the medium was adjusted to 7.0 with 1 M NaOH and the medium was distributed in flasks each containing 50 mL before autoclaving. Glucose was sterilized separately and added to the cultivation medium before inoculation.

**Alginate beads preparation:** The immobilized cell beads were prepared as follow: 80 mL of distilled water (25°C) were blended with 4 g sodium alginate in a blender at high speed and the obtained solution was sterilized by autoclaving at 121°C for 15 min. The predetermined weight of wet cells cultured in vegetative medium for 96 h at 30°C was suspended in 20 mL of sterile water and mixed with 80 mL of autoclaved sodium alginate solution under aseptic conditions. The obtained mixture was added drop wise to a cold 3% CaCl₂ solution with gentle stirring and allowed to solidify at room temperature and then stored overnight at 5°C. After hardening time, the beads were washed 3 times using sterile saline solution. All these steps were carried out under aseptic conditions.

**Determination of alginate bead diameter:** The average diameter of the beads were determined according to the method of Nguyen and Luong (1986), which depends on direct observation as follows: A magnifying glass was used to measure the diameter of a number of randomly chosen beads (50 beads). The beads were observed to ensure that all beads are spherical and uniform.

**Shake flask cultivation condition:** The cultivation was carried out in 250 mL Erlenmeyer flasks, each containing 50 mL vegetative medium. The flasks were sterilized at 121°C for 20 min. After cooling, the media were inoculated with immobilized cell beads and the inoculated flasks were agitated on a rotary shaker at 200 rpm and 30°C.

**Analysis**

**Sample preparation:** During the cultivation in shake flask, samples in the form of three flasks each withdrawn intermittently for analysis. Alginate beads were separated from the supernatant media and used for cell dry weight determination. The supernatant was centrifuged and used immediately for rifamycins and glucose determination.
Determination of cell dry weight (entrapped cells) in alginate beads: A number of randomly chosen beads (50 beads) were taken and washed thoroughly with distilled water and then dried at 110°C for a constant weight. The weight of entrapped cells was measured by calculation of the difference between the weight of immobilized beads before and after cultivation.

Determination of rifamycins activity: The rifamycin B and SV were determined according to the method of Pasqualucci et al. (1970). This method could be summarized as follows: samples were prepared for assay by taking 1 mL of cell-free fermentative broth and diluted 1:6 by acetate buffer adjusted to pH 4.64 (in case of sample) or acetate buffer solution containing 0.1% NaNO₃ (in case of blank). After shaking for 5 min, the optical density of the samples were measured colorimetrically using a double beam spectrophotometer at 425 nm for rifamycin B and at 447 nm for rifamycin SV against a blank solution. The concentrations of rifamycins were determined from a standard curve made by using authentic samples of rifamycins B and SV as standard.

Determination of glucose: Glucose concentration in the fermentation media was determined enzymatically by using glucose kits (RANDOX laboratories Ltd., Ardmore, United Kingdom).

RESULTS AND DISCUSSION

Effect of different concentrations of sodium alginate on rifamycin production: The aim of this experiment was to select the most suitable concentration of sodium alginate for the immobilization of the used strain. Different concentrations of 3.0, 4.0 and 5.0% sodium alginate (Fluka, special grade for cell immobilization) were used. At the end of the incubation period, the beads were washed 3 times with saline solution (0.9% NaCl) and allowed to dry at 110°C till a constant weight. The amount of rifamycins and entrapped cells were determined.

The results in Fig. 1 showed that, the production of both rifamycins B and SV was increased as the alginate concentration increased from 3 to 4% and decreased again upon increasing the alginate concentration up to 5%. On increasing alginate concentration from 4 to 5%, the rifamycins B and SV decreased from 544 to 450 mg L⁻¹ and from 534 to 363 mg L⁻¹, respectively. On the other hand, cell dry weight was decreased from 11.05 to 8.2 g L⁻¹ upon increasing the alginate concentration from 3 to 5%. The results indicated that, low concentration of alginate (not more than 4%) afforded better yields of rifamycins B and SV. This probably is due to the better diffusion of nutrients into the beads and the release of the products out of the beads (Tanaka et al., 1984; Kahlon et al., 1989) at this concentration as well as, the increase of gas exchange through the gel matrix (Matsunaga et al., 1980). The adverse effect of alginate concentration on rifamycin production was, however, much more pronounced in the higher level. On increasing alginate concentration from 4 to 5%, the production of rifamycins decreased due to mass transfer limitation and hindrance of oxygen diffusion (Nasri et al., 1989; Oyaas et al., 1995). These results were also in accordance with the fact of hindrance of oxygen diffusion due to high alginate gel concentration which causes lower gel porosity (Omar et al., 1993; Pilkington et al., 1998).

Effect of different bead diameters on rifamycin production: This experiment was done using cells immobilized in 4% alginate beads of different diameters: 3.0, 3.7 and 4.2 mm. The beads preparations were carried out by using sterile pipettes of different orifice diameters.
As shown in Fig. 2, on increasing bead diameter from 3 to 4.2 mm, the rifamycins B and SV increased from 432.75 to 565 and from 419.5 to 589 mg L\(^{-1}\), respectively. The cell dry weight also was increased from 7.95 to reach 10.7 g L\(^{-1}\) upon increasing the bead diameter from 3 to 4.2 mm. Therefore, out of three diameters investigated, beads of 4.2 mm diameter were the most suitable for the production of rifamycins. However, the volumetric increase in rifamycins production was due to the increase in biomass rather than the increase in cell productivity since there were no significant differences in antibiotic specific yield. The specific yield of rifamycin B production (calculated as mg rifamycin B per g cells) were 54.4, 55.7 and 52.8 mg g\(^{-1}\) for 3, 3.7 and 4 mm beads diameter, respectively. The influence of alginate bead diameter was studies by Idris and Suzana (2006) for lactic acid production and they found that increasing bead diameter from 1 to 5 mm decreased volumetric lactic acid production but this study didn’t gave any result on the relative yield.

**Effect of different inoculum ages on rifamycin production:** This experiment was carried out to investigate the effect of the age of inoculum on the production of rifamycin. Different ages of inoculum ranged from 72 to 168 h vegetative cells were immobilized in alginate beads, as described before, were used as inoculum. The results in Fig. 3 showed that, the production of both rifamycins B and SV was increased as the inoculum age increased up to 96 h. On increasing the inoculum age from 96 to 168 h, antibiotic production decreased from 668 to 267.5 mg L\(^{-1}\) and from 542.5 to 234.5 mg L\(^{-1}\), for rifamycin B and SV respectively. The cell dry weight was also increased from 8.5 to 9.55 g L\(^{-1}\) upon increasing the age of inoculum from 72 to 96 h and started to decrease again till it reached 5.7 g L\(^{-1}\) when the inoculum age was 168 h. However, the age of inoculum is usually a critical factor for antibiotic production. Since the immobilization arrest or decrease the cell growth, the phase of cell growth prior immobilization will influence the cell productivity significantly. Thus, we can conclude that immobilization of cells previously grown for 96 h in vegetative culture were the most active inoculum. This conclusion was also confirmed by the glucose concentration data since the maximal glucose consumption (the lowest residual glucose concentration) was obtained in this culture.
Effect of different bead numbers on rifamycin production

These investigations were achieved by inoculating the fermentation medium with different number of beads: 50, 70, 90, 110 and 130 beads per 50 mL culture. The results in Fig. 4 showed that, the production of both rifamycins B and SV was increased as the bead number increased. On increasing the bead number from 50 to 130 beads, the rifamycins B and SV increased from 322.5 to 719.5 mg L\(^{-1}\) and from 302.5 to 561 mg L\(^{-1}\), respectively. The cell dry weight was also increased from 6 to 12.45 g L\(^{-1}\) upon increasing the bead number from 50 to 130 beads per 50 mL fermentation broth. To investigate whether this increase in volumetric activity was due to the increase in inoculum size or cell activity. The specific rifamycins production (mg rifamycin per g cell) was calculated. The results showed that the specific rifamycins B and SV production was increased by increasing the bead number up to the number equivalent to 40 mg per flask (90 beads) beyond this level the specific antibiotic production decreased significantly.

The maximal specific antibiotic production of 70 mg g\(^{-1}\) for rifamycin B and 57 mg g\(^{-1}\) for rifamycin SV was obtained in flasks inoculated by 90 beads. Farid et al. (1995) showed also that the maximal rifamycins specific production was also achieved using immobilized cells of 40 mg cells as inoculum. By increasing the bead number in the fermentative media over a certain limit, some metabolic changes apparently increased. These metabolic changes include: reduced biomass yield, decreased specific growth rates, increased rates of glucose consumption, changes in surface tension and changes in osmotic pressure (Vijayalakshmi et al., 1979). All these metabolic changes explained the several folds decrease in the oxygen uptake rate, which caused decreasing in the rifamycins yields at high inoculum size.

Effect of different cell loadings on rifamycin production:

Different concentrations of cells in alginate solution were prepared. The concentration of the vegetative inoculum cells were (4.55 g cells L\(^{-1}\) alginate\(^{-1}\)), (9.1 g cells L\(^{-1}\) alginate\(^{-1}\)), (13.65 g cells L\(^{-1}\) alginate\(^{-1}\)) and (18.2 g cells L\(^{-1}\) alginate\(^{-1}\)) of precultured cells. The number of beads used was 90 beads. The results in Fig. 5 showed that the production of both rifamycins B and SV was increased as the cell loading increased from 4.55 g cells L\(^{-1}\) alginate\(^{-1}\) up to 9.1 g cells L\(^{-1}\) alginate\(^{-1}\). The rifamycins B and SV production reached 656 and 559.5 mg L\(^{-1}\), respectively. Further increase in cell loading decreased the antibiotic production. The cell dry weight was also increased from 6 to 13.6 g L\(^{-1}\) upon increasing the cell loading from 4.55 to 18.2 g cells L\(^{-1}\) alginate\(^{-1}\). On increasing the cell loading from 4.55 to 9.1 g cells L\(^{-1}\) alginate\(^{-1}\), the production of rifamycins was increased by 49.54 and 38.06% for rifamycins B and SV, respectively. Thus, the productivity was increased on using high cell loading beads. The maximum production of rifamycins was however achieved at 9.1 g cells L\(^{-1}\) alginate\(^{-1}\). When the viable cell concentration increased inside the alginate matrix to exceed a certain limit, the shape of immobilized cells may have been distorted and/or their volume reduced. However, this would have lead to an increase in the packing or cell density above the limit, which was often assumed for freely suspended cells. This could be attributed to the pressure exerted from other cells and the immobilization matrix (Stewart et al., 1989). Disturbances in the growth pattern of immobilized cells due to contact with the immobilization carrier or other cells are also proposed (Ohmoro et al., 1983; Doran et al., 1986). This also reflects directly in the cell physiology and productivity.

Effect of alginate gel modification on the production of rifamycins by immobilized cells: Different hardening agents were used before bead formation such as gum
Fig. 5: Effect of different cell loading inside the alginate beads on rifamycins production

 Cabrera and Gellman (2002) arabic and gellan gum to increase the stability of alginate beads prior fermentation. The results in Fig. 6 showed that the production of both rifamycins B and SV was increased by using gellan-gum as a hardening agent before bead formation compared to pure alginate beads. The rifamycins B and SV increased from 685 to 810 mg L⁻¹ and from 547.5 to 702 mg L⁻¹, respectively by using gellan gum modified beads compared to the non-treated beads. On the other hand, the antibiotic concentration decreased by using gum arabic modified beads. Stabilization of calcium alginate beads can be achieved by using additional cross-linking chemicals that are not influenced by chelators or exchangers. In particular, alginate can be cross-linked with gellan gum and gum arabic at very low concentrations (0.5 g L⁻¹) of both chemicals with sodium alginate before bead formation. This will result in the production of more stable and lower porosity complexes with improved leakage characteristics (Smidsrød et al., 1990). Gellan gum is a linear tetrasaccharide, which is consisting of repeating glucose, rhamnose and glucuronic acid units. Stronger alginate structure with gellan gum may explain the reduction in cell mass compared to pure alginate beads and also the significant increase in both volumetric and specific rifamycins yield. However, the use of different additives to alginate before immobilization to increase beads stability was also studied by different authors. The addition of xantan gum to alginate solution before immobilization increased the beads mechanical stability and was good matrix for urease enzyme immobilization (Elcin, 1995).

Bajpai et al. (2006) reported a significant improvement in bead stability of barium alginate-carboxy methyl guar gum. Another alginate hybrid beads with high stability composed of PVA-alginate complex for immobilization of Acidithiobacillus ferrooxidans was also reported recently (Yuji et al., 2006).

Repeate batch production of rifamycins by immobilized cells in alginate-gellan) beads for batch times of 72 and 144 h: The aim of this experiment was to investigate the ability of using the A. mediterranei cells immobilized in modified alginate (alginate-gellan gum) beads for the production of rifamycins and the effect of prolonged
cultivation time on the beads stability. However, two sets of experiments were carried out of different batch time of 72 and 144 h. The results in Fig. 7 and 8 showed that, the fermentation process was repeated five times successfully using the modified alginate beads with gellan gum. The first batch was 144 h in both cases to achieve high cell density inside the beads and to start the antibiotic production. The production of rifamycin B increased gradually during the first two batches. And decreased for the last three batches. On the other hand, the production of rifamycin SV decreased gradually starting from the first batch. Glucose consumption increased during the first batch and its concentration reached 16 g L⁻¹ for 72 h batch time and 15 g L⁻¹ for 144 h batch time. The concentrations of residual glucose in culture were less in subsequent batches. In general the beads were stable and no breaking of the gel beads was observed throughout the experiment time. The total amount of rifamycins B and SV during the five batches of 72 h were 3785 and 2395 mg L⁻¹, respectively. The total amount of rifamycin B and SV during the five batches of 144 h were 3360 and 2309 mg L⁻¹, respectively. Thus, there was no significant reduction in rifamycins production on reducing the batch time from 144 h to only 72 h. On the other hand, the decrease in the productivity of the immobilized cells during the repeated batch process in both cultures may be due to the proliferation of cells. The cells in the outer layers tend to prevent the adequate transfer of the substances to and from the inner layers (Riley et al., 1999). As a result, the cells in the inner layers died and resulted in decrease in the mass active productive cells.

CONCLUSION

The production of rifamycins by immobilized cells immobilized in alginate-gellan gum capsule was a good alternative cultivation system compared to the traditional free cell cultivation with significant reduction of batch time to only 72 h in case of repeated batch operation. Moreover, immobilization offers several potential advantages of a process of engineering nature to the fermentation system. These include ease of handling and
of cell separation, lowering of bulk viscosity, as well as the obvious potential benefits of increased cell concentration.

REFERENCES


