Short Communication

STUDIES ON RIFAMYCIN OXIDASE IMMOBILIZED ON AGAR GEL

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Rifamycin B, a microbial product of *Nocardia mediterranei* (10) is chemically converted to rifamycin S which acts as a starting compound for the production of rifampicin. Rifampicin is a successful antituberculosic and antileprotic drug (5, 13). The conventional chemical process which transforms rifamycin B to rifamycin S involves an organic solvent system and an artificial oxidant under acidic conditions, resulting in unsatisfactory low yields for industrial applications (11). Rifamycin oxidase, an intracellular enzyme of *Hemicola* and *Monocillium* spp. (6) and an extracellular enzyme of *Curvularia lunata* (14) can bring about the transformation of the microbial product rifamycin B to rifamycin S. However, immobilized enzymes have distinct advantages over free enzymes in terms of higher thermostability, reusability, operational stability and productivity. Elimination of the possible allergenic effects of the enzyme in the pharmaceutical industry is also an additional advantage of immobilization. The acetone-defatted cells of *Hemicola* sp. with rifamycin oxidase activity was immobilized by co-polymerization with acrylamide (7) and on cellulose acetate (4). Attempts have also been made to immobilize rifamycin oxidase of *C. lunata* on nylon fibers (15). This paper describes the properties of rifamycin oxidase of *C. lunata*, immobilized on agar gel and it also deals with the transformation of rifamycin B to rifamycin S by the immobilized enzyme preparation and its reusability. Rifamycin oxidase of *C. lunata* var. *aeria* was immobilized on agar gel. The pH and temperature optima of the agar immobilized enzyme preparation were 6.0 and 50°C, respectively. Thermostability of the immobilized enzyme preparation was checked at 30 and 40°C and it was found that on immobilization, thermostability had increased. Transforma-

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tion reaction was carried out with the immobilized enzyme preparation. Reusability studies with the immobilized enzyme preparation showed that diffusional resistances with smaller size (1 mm) beads were less compared to the higher size beads (2 or 2.5 mm).

Rifamycin oxidase was obtained by cultivating *C. lunata* as described previously (3). The enzyme was partially purified by using a Sephadex G-75 column equilibrated in 0.1 M phosphate buffer pH 6.5. The partially purified rifamycin oxidase was immobilized on agar by the method of Matsunaga et al. (8). Five milliliters concentrated enzyme was added to agar solution (200 mg agar were dissolved in 5 ml of 0.9% NaCl solution by heating at 100°C and then cooling at 50°C) in a beaker which was kept at 50°C water bath with constant stirring. After the enzyme addition was completed, the solution was immediately poured on a big petri plate and allowed to cool at 4°C for half an hour. The gel was cut into desired pieces and washed with phosphate buffer (0.1 M, pH 6.5) until the significant amounts of enzyme activity were no longer detected in the wash solution.

Enzyme activity was measured using the method of Seong et al. (12). Agar immobilized enzyme (100 mg) was added to 2 ml of 2 mM rifamycin B solution. After incubation at 50°C for 1 h, 3 ml of 1:1 methanol:phosphate buffer was added. The mixture was boiled for 3 min in a boiling water bath, centrifuged for 5 min at 4,000 rpm and the absorbance of the supernatant was measured at 525 nm. One unit of enzyme activity was defined as an equivalent of 1 μ mol of rifamycin S formed per milligram of immobilized enzyme preparation in 1 h under the specified conditions. Rifamycin B concentration was measured using the method of Pasqualucci et al. (9). The absorbance of the diluted solution was measured at 425 nm in a 1 cm cell against the other set of the same diluted solution oxidized by 0.1% (w/v) NaNO₂. The rifamycin B concentration in the transformed broth was calculated using the following formula:

$$\text{Rifamycin B } (\mu\text{g ml}^{-1}) = \frac{A_{425} \times 50,000}{21.5},$$

where A_{425} is the absorbance at 425 nm and 21.5 is the absorptivity of rifamycin B. The method described by Chung et al. (4) was used for the estimation of rifamycin S. Two ml of the sample was diluted with 4 ml of methanol and 4 ml of 0.1 M phosphate buffer (pH 6.5) and the solution was hydrolyzed from rifamycin O to rifamycin S by boiling for 3 min. The absorbance was read at 525 nm with appropriate buffer blank.

The optimum pH and temperature for rifamycin oxidase activity immobilized on agar gel were 6.0 and 50°C, respectively. On immobilization, the optimum temperature did not change and it was the same as reported for soluble enzyme (14).

To find out the thermostability of the agar immobilized enzyme preparation, the catalysts were incubated in phosphate buffer (0.1 M, pH 6) at 30 and 40°C up to 16–28 days. From time to time, a few beads were taken out and enzyme activity

Fig. 1

Fig. 2

Fig. 1. Thermostability of agar immobilized rifamycin oxidase activity of *C. lunata*.

Fig. 2. Transformation of rifamycin B with agar immobilized rifamycin oxidase of *C. lunata*.

Fig. 3. Reusability of agar immobilized rifamycin oxidase of *C. lunata*.

Transformation time is plotted against the number of cycles used when percentage of transformation was kept constant.

was measured. It is evident from Fig. 1 that the agar immobilized rifamycin oxidase has half-lives of 24 and 9 days at 30 and 40°C, respectively. In comparison with the free enzyme, immobilized enzyme preparation has longer half-lives at 30 and 40°C. At 30°C, agar immobilized enzyme has a half-life of 24 days while free enzyme at this temperature has a half-life of 9 days only (1). Transformation experiments with the immobilized enzyme preparation were carried out in shake flask. A few immobilized beads (size 2.5 mm) were taken in 250 ml flask containing

25 ml of 10 g/l of rifamycin B solution. The flasks were then incubated at 28°C with a rotational speed of 200 rpm. Samples were withdrawn from time to time and estimated for rifamycin B and rifamycin S. Agar immobilized beads were strong enough for repeated use; however, it took a longer time of 12 h to transform rifamycin B. As illustrated in Fig. 2 the initial rate of transformation was very low and during the initial 2 h, there was no production of rifamycin B. The yield of rifamycin S was not stoichiometrically related with rifamycin B. The time required for the transformation of 10 g/l of rifamycin B with soluble enzyme was reported to be 3 h (2).

The reusability of immobilized rifamycin oxidase was studied by keeping the percentage of transformation constant. It is seen from Fig. 3 that with the agar immobilized enzyme preparation (bead size 2.5 mm), the time required for the full transformation of rifamycin B to rifamycin S between 13–15 h up to two cycles of use. The longer time of transformation with the number of reuse, may be the leaching out of the enzyme from the gels or the deactivation of the enzyme due to the prolonged incubation and agitation. The immobilized enzyme preparation was quite stable at 28°C (half-life at 30°C was 24 days) and the possible loss of enzyme by leaching was discounted as much less activity was detected in the supernatant. Next, to find out the diffusional limitations, experiments were carried out with a reduced size of beads (1 and 2 mm). It is also clear from Fig. 3 that the time required for the transformation of rifamycin B had decreased with the reduced size of beads. With the bead size of 2 mm the effect was not so predominant but the diffusional resistances reduced with 1 mm bead and in the 3rd cycle of reuse it took 11 h to complete the transformation.

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