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Immobilization and Characterization of Rifamycin B Oxidase in Cellulose Acetate Beads

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셀룰로오스 아세테이트에 고정화된 리파마이신 B 산화효소의 특성

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Rifamycin B oxidase converts rifamycin B to rifamycin S using oxygen as cosubstrate. *Humicola* spp. (ATCC 20620) was treated with acetone and the cell powder was immobilized with cellulose acetate. The properties of the immobilized enzyme was examined.

The optimum pHs of the immobilized and the free enzymes were 7.2. The optimum temperature of the immobilized enzyme was at 50-55°C, which was 5°C higher than that of the free enzyme. The activities of the immobilized enzyme appeared less sensistive with respect to the changes of temperature and pH as compared to those of the free enzyme. Twenty percent of the enzyme activity was recovered when the enzyme was immobilized in 3mm beads. The storage stability was good below 40°C, but the activity decreased very rapidly above 50°C. The physical strengh of the beads was good and was suitable as packing material in a three-phase enzyme reactor.

Rifamycin S is the key intermediate of antituberculosis agent rifampicin and is obtained commercially by chemical conversion of rifamycin B which is produced by fermentation using *Nocardia mediterranei* (1). However, the chemical conversion of rifamycin S requires organic solvent and oxidizing agent. Furthermore, the reaction is carried out in a strong acidic environment and can be quite corrosive to the reactor. In order to circumvent these difficulties biological conversion was sought. *Humicola* spp. (ATTCC 20620) and *Moncillium* spp. (ATCC 20621) were reported to have rifamycin B oxidase activity and the reaction mechanism was suggested (2). The effects of pH and glucose on the production of rifamycin oxidase were studied (3). The effects of pH and temperature of the enzyme immobilized in polyacrylamide gel were studied (4).

Since the biological oxidation reaction requires oxygen as cosubstrate, we need a reactor where good contacts among the gas, the substrate in the liquid phase and the immobilized enzyme can be made. Packed bed reactors are not suitable for this purpose. Fluidized bed and recently developed rotating packed disk reactor (5) are the types that can be considered. In order for the immobilized enzyme to be used in such reactors the physical strength of the immobilized enzyme should be strong enough to resist long-term abrasion in the reactor. Calcium alginate and polyacrylamide gels are too weak for such a purpose. Thus we immobilize the enzyme in cellulose acetate beads which have very good physical strength and its properties.

Materials and Methods

Materials and Equipment

Yeast-extract and bacto-peptone were obtained from Difco Lab.; glucose from Sigma Chemical Co.. Cellulose acetate was the product of Eastman Kodak. Rifamycin B was purified from the fermentation broth supplied by Yuhan Chemical Co., (Seoul, Korea). Other chemicals were of extra pure grade. Two-liter jar fermentor (N.B.S. Model C30) was used for the cell cultivation.

Methods

Humicola spp. (ATCC 20620) was cultivated in a medium containing glucose (1%), bactopeptone (1%), yeast extract (1%), CuSO4 (0.0025%). The medium was adjusted to pH 7 with 1N NaOH. The seed culture was grown in a shaking incubator at 200 rpm, 30°C for 2 days. The seed culture volume used for inoculation into a main fermenter comprised 5% of the total liquid volume (1l) in the jar fermenter. Main culture was carried out at 30°C at an agitation speed of 500 rpm, and aeration rate of 1vvm.

The cultivated cells were centrifuged at 5000 rpm and the harvested cells were stirred in acetone for 1-2 hours. The acetone-treated cells were filtered and air-dried at room temperature for 4-5 hours. The white cell powder obtained in this manner was stored in a refrigerator and was used as the enzyme.

Cellulose acetate was used as a carrier. Cellulose acetate (5 gr) was dissolved for 2 hours in a mixture of acetone (30 ml) and dimethylsulfoxide (20 ml). One gram of the cell powder was added to the solution followed by rapid mixing for uniform distribution. This solution was added dropwise in 0.1M phosphate buffer (pH 7.2) to form porous beads. The beads suspended in 0.1 M phosphate buffer was stored in a refrigerator for 1 day.

Rifamycin S was measured spectrophotometrically at 525nm. Two mililiter of the sample were diluted with 4 mL of methanol and 4 mL of 0.1 M phosphate buffer (pH 7.2) and the solution was hydrolyzed from rifamycin 0 to rifamycin S by boiling for 3 minutes. For measuring the activity the immobilized enzyme were suspended in a oxygen saturated buffer solution and stirred at 250 rpm. The initial reaction rate was measured by the amount of rifamycin S formed for 20 minutes at 50°C.

Results and Discussion

Effect of pH and Temperature

The effect of pH on the enzyme activity is shown in Figure 1. Phosphate buffer (0.1M) was used in the pH ranges

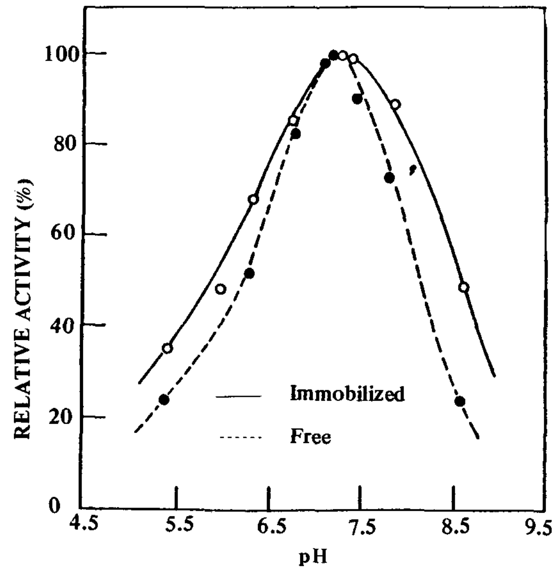

Fig. 1 Effect of pH on free and immobilized enzyme.

of 5.6-8.0, Glycine-NaOH buffer was used at pH 8.6. The optimum pH of the immobilized and free enzymes appeared at pH 7.2 and the pH of the former appeared broader than the latter. This phenomenon may be explained by the diffusional masking in the support (6). The effect of temperature was studied at pH 7.2 (0.1M phosphate buffer). As shown in Figure 2, the immobilized enzyme demonstrated the optimum temperature at 50-55°C while the free enzyme at 45-50°C. Thus the immobilized enzyme appeared more stable.

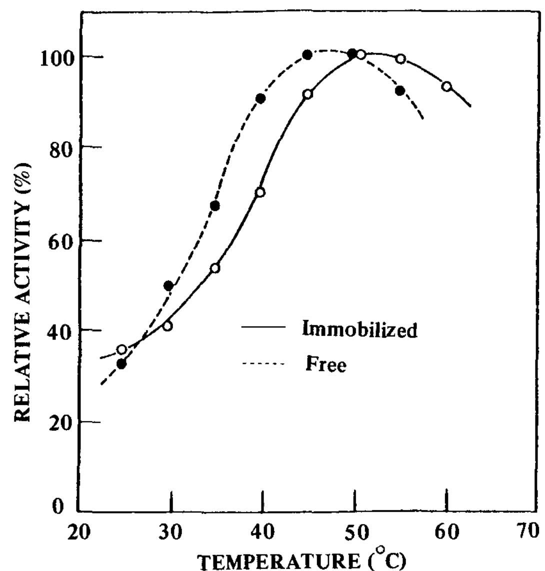

Fig. 2 Effect of temperature on free and immobilized enzyme

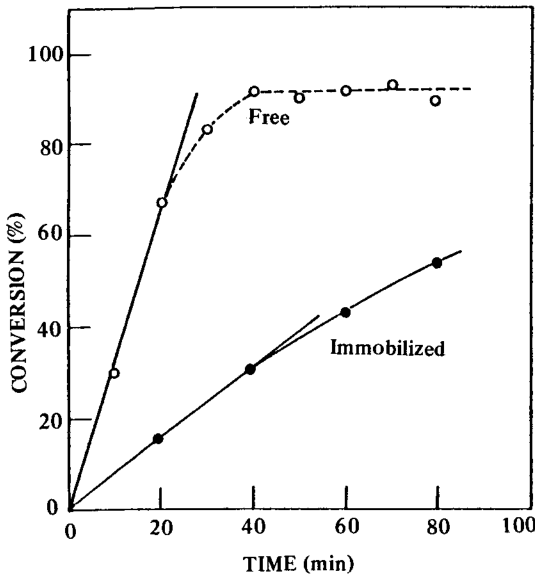

Fig. 3 Degree of conversion of rifamycin B by free and immobilized enzyme.

Recovery of activity

Figure 3 shows the degree of conversion of the free enzyme (0.1g) and the corresponding amount of the immobilized enzyme (wet wt. = 4.5g, rough bead diameter = 3mm) suspended a flask with 25mL of 1mM rifamycin B. The ratio of slopes represents the activity recovery, which is about 20%. Recovery of the enzyme activity increases as immobilized enzyme beads becomes smaller. Decrease of the enzyme activity during the immobilization process can be attributed to the following two factors; one by the enzyme denaturation by the immobilization reagents and the other by the mass transfer resistance in the carrier. It was observed that the immobilized enzyme beads consist of a thin smooth outer layer and a porous inner layer. It is, thus, conceivable that the major mass transfer resistance occurs in the outer layer.

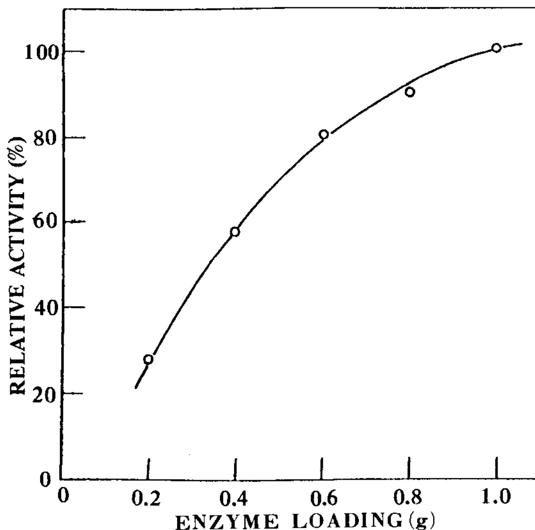

Fig. 4 Effect of enzyme loading on the activity of immobilized enzyme

Effect of Enzyme Loading

Figure 4 shows the variation of activities with enzyme loading. It was demonstrated that the optimal loading amount of the acetone treated cell powder was 1 gram. The enzyme loading more than 1 gram was not practical because of the precipitation of acetone treated cell powder during the immobilization process and thus it was difficult to maintain homogeneity.

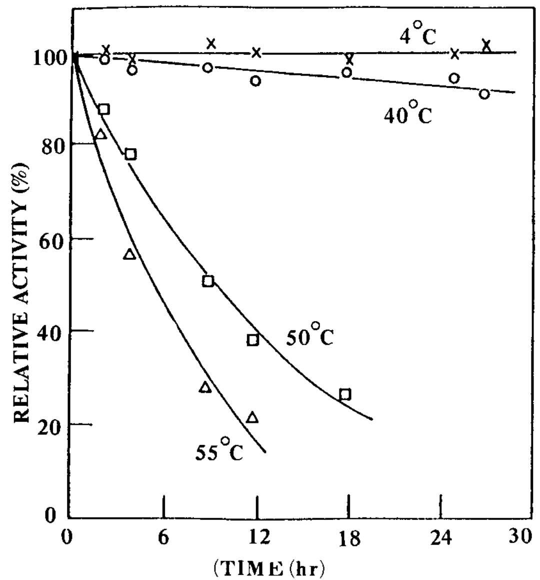

Fig. 5 Thermal stabilıty of immobilized enzyme

Storage stability

Figure 5 shows the thermal stability of the immobilized enzyme. There was no apparent decrease of the enzyme activity in the temperature range between 4°C and 40°C. It was observed that about 90% of the activity was retained after 27 hours at 40°C. However, the activity decreased rapidly at the temperature above 50°C.

ACKNOWLEDGEMENT

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요 약

리파마이신 B 산화효소의 활성을 갖고 있는 *Hu-*

micola spp. (ATCC 20620)를 아세톤 처리 한 후 cellulose acetate로 고정화하여 그 특성을 조사하였다. 고정화 효소에 있어서 pH는 7.2온도는 50~55℃에서 최대 활성을 보였으며, 비고정화 효소에 비하여 pH와 온도변화에 덜 민감하였다. 외경 3 mm bead size에서 20%의 활성회수율을 보였으며, storage stability는 40℃ 이하에서는 아주 좋았으며 50℃이상에서는 급격히 활성이 감소하였다. 고정화 담체로서 cellulose acetate는 물리적으로 견고하여 삼상반응기의 packing material로써 적당하였다.

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