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THYMIDINE PHOSPHORYLASE ACTIVITY OF ANUCLEATE MINICELLS OF *ESCHERICHIA COLI* IMMOBILIZED IN AN AGAROSE GEL MATRIX

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ABSTRACT

Escherichia coli whole cells, and minicells were immobilized on columns in an agarose gel matrix and evaluated for their thymidine phosphorylase (TPP) activity. The greatest specific activity of TPP was found with the induced minicells (2.2 µmoles thymine/min/mg protein) at a column retention time of 11 min.

INTRODUCTION

Industrial application of immobilized microbial cells is currently of interest and continuous production of several useful compounds such as aspartic acid (Chibata *et al.*, 1974; Tosa *et al.*, 1974; Sato *et al.*, 1975), l-citrulline (Yamamoto *et al.*, 1974a), urocanic acid (Yamamoto *et al.*, 1974b; Jack and Zajic, 1977), 6-amino penicillanic acid (Sato *et al.*, 1976) and L-malic acid (Yamamoto *et al.*, 1976; Takata *et al.*, 1979) have been accomplished using microbial cells in polyacrylamide gel lattices. Apart from toxicity problems associated with the support system, a major disadvantage in using whole cell systems in the immobilized state is the need to sustain activity while minimizing cell growth (Mosbach 1976; Chibata and Tosa 1977).

A novel microbial system which may be useful in bioconversion reactions involves the use of cell division mutants of *Escherichia coli* which produce anucleated minicells (AMCs). AMCs have general utility in molecular and applied microbiology (Frazer and Curtiss, 1975, Khachatourians, 1976). In this paper we report for the first time the utility of anucleated minicells (AMCs) in an immobilized state for the biological interconversion of nucleosides. Some of these results were described earlier (Khachatourians *et al.*, 1981).

MATERIALS AND METHODS

Chemicals and Reagents: Agarose (standard low-m_r) was obtained from Bio-Rad Laboratories, Richmond, Ca. Thymidine was from Sigma Chem. Co., St. Louis, Mo. All other chemicals were also reagent grade.

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BACTERIAL STRAINS

Escherichia coli strains GK 100 or 402 were used throughout this study. For the whole cell preparations the non-minicell producing strain (GK 100) of *E. coli* and for whole cells or AMCs the minicell producing strain (GK 402) of *E. coli* were used.

MEDIUM AND CULTURE CONDITIONS

For cell growth or AMC production the bacterial strains were grown in a defined salts medium (Khachatourians *et al.*, 1974). Thymidine at a final concentration of 200 µg/ml was used for the induction of TPP activity. Cultures were grown in shake flasks at 37°C in a water-bath shaker, 200 rpm speed (New Brunswick Scientific, N.J.) and harvested after 14-16 hr incubation. The AMCs were harvested and purified from the cultures of strain GK-402 by a combination of centrifugation and ultrasound as described before (Khachatourians and Saunders, 1973; Khachatourians *et al.*, 1974).

IMMOBILIZATION PROCEDURE

Twenty ml of a 1.0 OD (600nm) suspension of AMCs (0.02 g dry wt) or 100 ml of a suspension, 1.9 O.D., or parental or whole cells (1.0 g dry wt) were centrifuged. Agarose (0.4 g/10 ml) was molten at boiling condition. The centrifuged cells were resuspended in 5.0 or 10 ml of cooling (50°C) agarose respectively, vortexed and allowed to gel. The resultant gel was cut (with knife) to particle size 3-5 mm^2 then blended in a Waring blender to a particle size 0.1 to 0.5 mm in diameter.

REACTOR CHARACTERISTICS

Immobilized minicells or parental cells were packed into columns (12 cm x 1.8 cm), and a solution of 1 mM thymidine (pH 6.3) was passed through the column at various flow rates (0.008 to 0.8 ml/min) at 37°C. The thymidine breakdown product in the effluent was monitored using the spectrophotometric assay method of Brosseau *et al.* (1981). Activity is defined as µmole of thymine/min retention or space time/mg cell protein. The space time is the volume (ml) of the reactor divided by the flow rate (ml/min).

RESULTS AND DISCUSSION

The AMCs are the end result of asymmetrical polar cell division in *E. coli* (Fig. 1). The production of AMC is controlled by the mechanism responsible for normal cell division (Khachatourians *et al.*, 1973). AMCs contain many metabolic enzymes and transport systems of the whole bacterial cells. We have studied the suitability of AMCs for immobilization in agarose and use in a bioreactor.

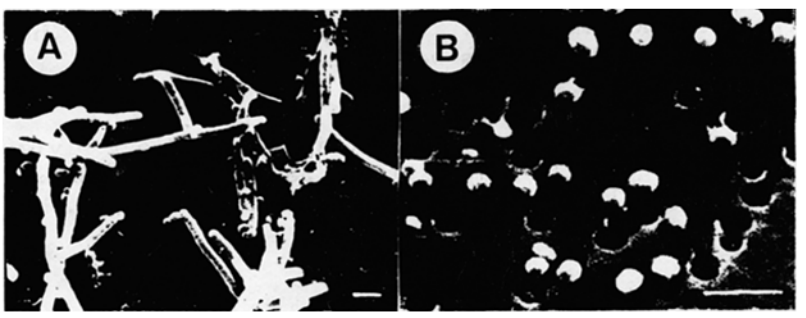

Figure 1. Scanning electron micrographs of whole bacterial cells (A) and anucleate minicells (B). Bar represents 2 µm.

Agarose was chosen over other gel matrices because it is non-toxic and does not require organic solvents (Chibata *et al.*, 1974; Larson and Mosbach, 1976) associated with entrapment in polyacrylamide, cross-linking by glutaraldehyde or encapsulation by polyurea (Chibata *et al.*, 1974; Shimiza *et al.*, 1975).

To monitor the biological activity of the immobilized AMCs we chose to study the activity of thymidine phosphorylase (EC.2.4.2.4.) which catalyses the phosphorolytic catabolism of thymidine with the formation of desoxyribose-1-phosphate and thymine. This enzyme segregates into AMCs, indicating its distribution throughout the periplasm and cytoplasm of *E. coli* cells (Khachatourians *et al.*, 1982). In AMCs from strain GK 402 the absence of DNA allows for a quantitative measurement of the bio-conversion reaction of thymidine. In whole cells, on the other hand, only the amount of one of the bioconversion products, thymine, in excess of the organism's requirements for DNA synthesis could be detected in the culture fluid. The lack of DNA in anucleate minicells suggests that thymidine phosphorylase activity by AMCs should result in an apparent higher recovery of thymine from the cells which have no additional requirement for the incorporation of this base unique to DNA.

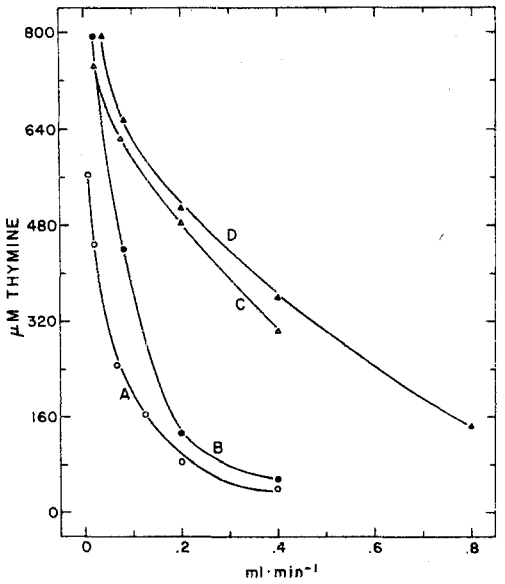

Figure 2. Yields of thymine (μM) against thymidine flow rate. (A) Non-induced parental cells, (B) induced parental cells, (C) induced AMC producing whole cells, (D) induced AMCs.

Thymine phosphorylase activity by either AMCs, by whole cells or by non-AMC producing parental cells immobilized in a column support system was measured at substrate flow rates from .008 to 0.8 ml/min (Fig. 2).

The effluent thymine concentration increased with decreasing flow rates. The complete conversion of the administered substrate at the concentration used was achieved only at the lowest flow rates used. The AMCs at similar optical densities to the parental cells in spite of their small size were approximately four times as active as the parental cells and a 50% substrate conversion rate was achieved at a flow rate of 0.4 ml/min. To achieve a similar conversion rate using parental cells required a substrate flow rate of 0.1 ml/min. Even at the maximum flow rate used (0.8 ml/min) a 20% conversion of the substrate was effected by the AMCs. The greatest TPP activity in immobilized minicell and whole cell activity was obtained at relatively fast thymidine flow rates. There may have been an inhibition of the reaction occurring over the slower flow rates; an increase in the flow rates, however, did not result in direct proportional decreases in thymidine conversions.

It can be seen from Fig. 2 that the immobilized whole cell systems have a greater potential for expression of induced TPP activities (curve B) than cells not previously exposed to thymidine (curve A). This is particularly evident at the slower flow rates. This difference in activities is not apparent at the faster flow rates used. This suggests that a low level of induction of enzyme synthesis occurred in the immobilized parental cells after long periods of exposure to thymidine despite the lack of required growth nutrients. The similarity between the whole cell data and AMC data can be attributed to the differences in the wild type (GK 100) and AMC producing (GK 402) strains.

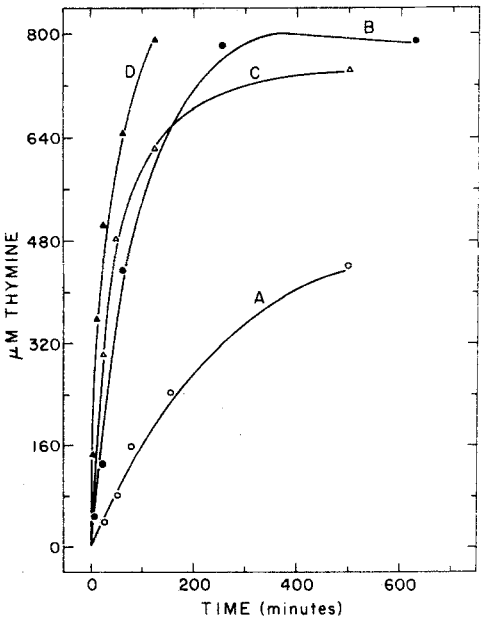

Figure 3. Comparison of thymine yields versus retention time. (A) Non-induced parental cells, (B) induced parental cells, (C) induced AMC producing whole cells, (D) induced AMCs.

Fig. 3 shows the effect of retention time on the yield of thymine as a result of TPP activity. Fig. 3 also shows the magnitude of the difference between the non-induced cells and the induced whole cells and AMC.

Maximum specific activity of the enzyme was obtained with induced AMCs, (2.2 μmoles thymine/min/mg protein) at a column retention time of 11 min. (Fig. 4). This figure is 13x higher than the batch figure quoted in Brosseau *et al.* (1981) with non-induced cells. The maximum productivity achieved with induced whole cells and parental cells was 0.5, 0.16 and 0.1 μmoles thymine/min/mg protein at retention times of 20 and 63 and 75 min. respectively.

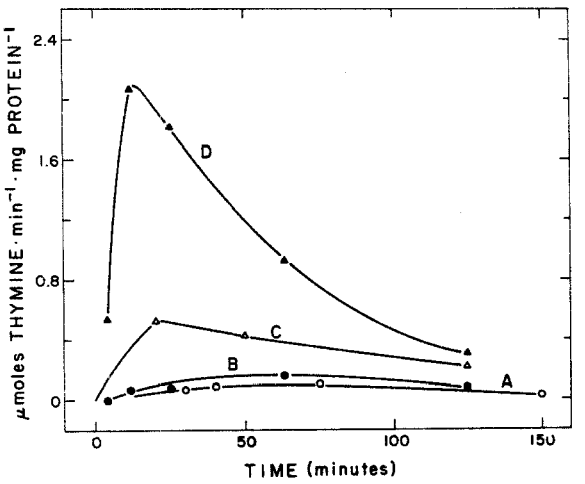

Figure 4. Productivity or specific activity of thymidine phosphorylase against retention time. (A) Non-induced parental cells. (B) induced parental cells, (C) induced AMC-producing whole cells, (D) induced AMCs.

In conclusion it has been shown that whole cells and anucleated minicells of *E. coli* can be immobilized by entrapment into an agarose gel matrix using a simple procedure. The AMCs can carry out simple biocatalytic reactions under batch as well as immobilized states. The stability of AMCs, the absence of growth and inherent problems of cell ageing, coupled with their enzymic activities make them prospective tools for continuous substrate transformation systems for quite long periods in immobilized columns. Further application of the immobilized AMCs is under study in this laboratory.

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