A SIMPLE METHOD FOR THE PRODUCTION OF HIGH TITRE PENICILLINASE

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The culture filtrates of certain penicillinase-constitutive mutant strains of *B. cereus*, grown overnight in casein hydrolysate and citrate, can serve, without further treatment, as a source of stable, high-titre penicillinase suitable for inactivation of penicillin in sterility tests, blood cultures, etc.

Many laboratories in hospitals, research institutes and pharmaceutical establishments need supplies of penicillinase from time to time, largely in order to permit valid sterility tests on penicillin preparations or for bacteriological investigations on body fluids of patients under treatment with penicillin. The enzyme is produced by a wide range of bacteria[1,2] in extremely variable quantities. In the genus *Bacillus*, which gives the highest yields, it is usually adaptive, and penicillin must be added to the culture for optimal production. Under many conditions the enzyme is unstable and penicillinase activity may be lost rapidly. For most purposes the amount required is so modest that high titres and stable preparations, though desirable, may not be essential. However, the recent isolation[3,4] of penicillinase-constitutive mutant strains of *B. cereus*, forming very large quantities of the enzyme extracellularly in casein hydrolysate without the need for stimulation with penicillin, has provided a means by which a stable, high titre preparation can be assured in any laboratory, at minimal cost in time and effort.

Technique

Strains, B. cereus 5/B (NCTC9946[3]) or *B. cereus* 569/H (NCTC9945[4]), both penicillinase-constitutive mutant strains. Strain 569/H produces higher titres than strain 5/B, but is genetically, perhaps rather less stable, and for most purposes strain 5/B is probably preferable.

Medium. Casein hydrolysate, Difco, technical grade 10 g.; KH_2PO_4, 2·72 g.; sodium citrate, 5·88 g. dissolved in 200 ml. of water; concentrated NaOH solution added to give pH 7·2, and water to 1 l. It is sterilised by heating in an autoclave. A portion (100 ml.) of a solution containing 0·41 g. $MgSO_4.7H_2O$ and 1 ml. of a 0·16 per cent solution of $(NH_4)_2$ $Fe(SO_4)_2$, 6 H_2O is autoclaved and added to the solution separately.

Production. Using an inoculum of washed spores[6], or vegetative cells from a nutrient agar slope, the culture is left standing at any convenient temperature (between 18° and 37°) until growth becomes visible whereupon it is shaken in a conical flask aerobically at 35° to 37° for approximately 16 hours overnight. Efficient aeration is essential for maximal enzyme formation. The cells are separated by centrifugation, and the supernatant fluid retained. If sterilisation is not required, the

culture supernatant can be preserved by the addition of a few crystals of oxine (8-hydroxyquinoline). For many purposes this may be undesirable, when the supernatant can be sterilised by filtration through an Oxoid membrane filter (Oxo Ltd.). This may involve 20 per cent loss in activity, through adsorption to the membrane. Sintered glass or Seitz filters may adsorb most of the enzyme, particularly with preparations obtained from cultures grown in casein hydrolysate, and are therefore unsatisfactory. The sterile filtrate should contain from 2000 to 10,000 units of enzyme per ml. This compares with penicillinase activities of 15, 25, 190, 380 and 1300 units/ml. respectively found in unconcentrated preparations dispensed by five leading pharmaceutical firms trading in this country. The unit of penicillinase, previously defined[7], is that amount which will hydrolyse one micromole of benzylpenicillin per hour at 30° and pH 7·0 under conditions where the enzyme is saturated with substrate: that is, for practical purposes, at a penicillin concentration above 1000 units/ml. One-tenth ml. of the filtrate, as prepared, should therefore be able to destroy between 1,000,000 and 5,000,000 units of benzylpenicillin per hour under these conditions.

This activity corresponds to a production of from 6 to 30 μg. of pure penicillinase per ml. of culture. This single enzyme may, in fact, constitute up to 30 per cent of the total extracellular protein formed by the cells. It can, if necessary, be isolated from the culture medium, and finally crystallised, by a procedure already described[4,5]. For many purposes, however, the culture supernatant has sufficient activity and is quite satisfactory, without further treatment.

The sterilised filtrate should be kept in the refrigerator where its activity is maintained at least for several weeks. Alternatively, it can be freeze-dried and sealed in ampoules, where it can be preserved without loss of activity, certainly for many months.

Loss of activity in solution is due largely (*a*) to hydrolysis of the enzyme by a proteinase which may also be present in the culture medium and (*b*) to adsorption on solid surfaces, particularly glass, for which penicillinase has an unusually high affinity[4]. However, the incorporation of citrate in the culture medium removes the Ca^{++} which is essential both for formation and function of bacterial proteinase[7,8]; this factor is not therefore one which, in this method, will cause any serious decrease in activity. Loss of activity due to adsorption on glass is liable to occur if the enzyme preparation is given opportunity for fresh contact with glass surfaces such as may occur on pipetting or transfer from one vessel to another particularly if it is diluted in water or aqueous buffer solution. This loss can be completely prevented by the incorporation of 1 per cent gelatin in the solution. This, however, may not always be desirable and might have to be omitted in which case a certain amount of loss on dilution may be inevitable. This will not be serious if the technique recommended here is adopted because the initial penicillinase titres are so high that the proportional loss of activity will be low.

The fact that the Michaelis affinity constant of this enzyme for benzylpenicillin is approximately 20 units/ml.[9] should be borne in mind when

calculating the quantity of enzyme needed for *complete* destruction of a given amount of penicillin in a given time. As soon as the penicillin concentration falls much below 1000 units/ml. the enzyme will cease to be saturated with its substrate and the rate of hydrolysis will begin to decrease appreciably. It is, therefore, suggested that, for this purpose, at least 10 times the amount of enzyme theoretically needed under conditions where the enzyme is always saturated with substrate, be in fact added in order to ensure that the reaction is complete in the time required.

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