Fermentation Monitoring by Polymeric-Resin-Based Microbial Assay

JAY J. SEATON and HENRY Y. WANG, *Department of Chemical Engineering, The University of Michigan, Ann Arbor, Michigan 48109*

Summary

A polymeric-resin-based microbiological assay is shown to yield results consistent with classical inhibition zone theory. This novel assay is applicable to cycloheximide, an antifungal antibiotic, at concentrations below 1.0 μg/mL and up to 800 μg/mL. Addition of ethanol to the cut-out wells of the assay plate helps to release the antibiotic and drastically increases the inhibition zone size. The antibiotic concentrations of both two component and multicomponent systems can be determined using this method. The use of polymeric-resin beads to monitor antibiotic concentrations is shown to be an effective technique for fermentation monitoring.

INTRODUCTION

The monitoring of fermentation processes is an essential element in their control and development. Screening programs that monitor the production profiles of mutants can be used to identify those mutants which show promise of providing an increased product yield. In this process, the final product concentration in the fermentation broth is generally used as a determinant of synthesis capacity. The timing of the monitoring assay can be of great importance in the determination of the final product concentration, for the relative product concentrations of mutants at any arbitrary time do not necessarily reflect their relative peak concentrations and volumetric productivities.

The solution to this problem is to either determine the complete profile of each mutant, with concurrent increases in manpower, equipment, and media costs, or to somehow monitor the maximum concentration achieved throughout the fermentation period.

Our approach to solving these problems involves extending a newly developed technique of resin-based microbiological assay[1] to the monitoring of product concentrations in screening vials that contain only 2–5 mL of fermentation broth. This method utilizes macroporous polymeric resin, in conjunction with the plate (agar diffusion) method of microbiological assay, to detect cycloheximide, a glutarimide antibiotic.[1] In addition to extending the assay to the monitoring of fermentations, we have modified it so that the inhibition zones are clearer and develop earlier than those of the previously

Biotechnology and Bioengineering, Vol. XXV, Pp. 771–779 (1983)
 CCC 0006-3592/030771-09$01.90

described method. Detection of cycloheximide was possible at concentrations as low as 0.1 μg/mL.[1]

MATERIALS AND METHODS

Organisms

The test organism for the microbial assay was *Saccharomyces cerevisiae* Y-139 (Northern Regional Research Laboratory, Peoria, IL). The organism for the cycloheximide fermentations was *Streptomyces griseus* UC 2132 (Upjohn, Kalamazoo, MI).

Media

The seed media for *S. griseus* and the fermentation broth used to produce cycloheximide from *S. griseus* had the same composition as that reported by Kominek.[2]

Treatment and Selection of Resins

The XAD-4, XAD-7, and XAD-8 resins (Rohm and Haas, Philadelphia, PA) were sieved to obtain beads in the 1-mm-diam range. These resin beads were soaked in methanol for 24 h before being rinsed in distilled water. The resin beads were then placed in distilled water and autoclaved for 15 min at 121°C, after which they were stored at room temperature in closed containers of distilled water.

Assay Standards

To prepare the aqueous standards, pure cycloheximide (Upjohn, Kalamazoo, MI) was dissolved in distilled water.

The whole broth and supernatant standards, with concentrations of cycloheximide from 0.10 to 2.0 μg/mL, were produced by adding the appropriate amount of 200 μg/mL aqueous standard to fermentation broth and its supernatant.

The whole broth and supernatant standards, with concentrations from 10 to 800 μg/mL, were produced by diluting the broth and supernatant of an *S. griseus* shake-flask fermentation in which the broth cycloheximide concentration had reached 830 μg/mL. The supernatant was obtained by certrifuging the broth for 10 min at 2400 rpm (Damon IEC UV centrifuge, MA).

Chemical Assay for Cycloheximide

The concentration of cycloheximide in the fermentation broth was determined using the method of Takeshita, Takahashi, and Kuda.[3] as modified by Kominek[2] for use with fermentation broth.

Resin-Based Microbiological Assay for Cycloheximide

Preparation of assay plates

The bioassay medium contained the same ingredients as that for the paper-disk plate method for cycloheximide.[4] After heating to dissolve the agar, the medium was sterilized for 15 min at 121°C. After cooling of this medium to 47°C, a 0.5 vol. % addition of the test organism was made. The test organism was a 24-h liquid culture of *Saccharomyces cerevisiae* NRRL-Y-139 with full viability. Ten-milliliter aliquots of this media were dispensed into 85-mm-diam plates. These assay plates were then refrigerated in sealed bags and the plates were used within two weeks.

Adsorption of cycloheximide

One resin bead of either XAD-4, XAD-7, or XAD-8 was placed in a screw-capped sample vial along with 5 mL of a test sample. These vials were then shaken in a 25°C incubation room on a rotary shaker (300 rpm) for 2 h. This time period is sufficient to ensure complete equilibration. At the end of the 2-h period the resin beads were removed from the vials and dried with a paper towel. As noted in the Results section, some resin beads were allowed to equilibrate with the broth of an ongoing vial fermentation for periods up to 11 days.

Resin assay

Beads in the amount of 1-5 (see the Results section) were placed in the previously cut well (diam = 3 mm) of an assay plate. A small amount of 95 vol. % ethanol was added to this well to aid in eluting the cycloheximide. These plates were then incubated for 24 h at 25°C. The inhibition zones that resulted were read with the use of a Fisher-Lilly antibiotic zone reader.

Fermentations

Shake flask

Fifty milliliters of *S. griseus* seed media (receipe referenced earlier) was inoculated and then incubated in 250-mL shake flasks at 300 rpm and 25°C for 48 h. Fifty milliliters of cycloheximide fermentation media (composition referenced earlier) was then inoculated with 2 mL of the 48-h seed culture and incubated in 250-mL shake flasks at 300 rpm and 25°C.

Small vial

The small-vial fermentations were run in cylindrical sterilizable glass screw-capped vials of ca. 10-mL total volume. Five milliliters of sterile fermentation media, which had been inoculated in the same manner as that

for shake flasks, were placed in each vial. After securely tightening the caps, they were incubated at 25°C on a rotary shaker (300 rpm). One bead of XAD-8 resin was added at the beginning or at points throughout the fermentation as described in the Results section.

RESULTS AND DISCUSSION

Assay Characterization

The amount of cycloheximide desorbed from the resin bead, as indicated by the size of the inhibition zone formed, is dependent on the amount of ethanol applied, as shown in Figure 1. The specified volumes of ethanol were obtained by successive additions to the wells over a period of 20 min. As indicated, the presence of up to 14 μL of ethanol will in itself produce no inhibition zones. Thus the inhibition zones are only a result of desorbed cycloheximide. The smallest zone sizes, obtained without the use of ethanol, resulted from the elution of cycloheximide from the resin bead by the water present in the agar. As the volume of applied ethanol was increased, the size of the inhibition zone also increased but addition of volumes greater than 10 μL produced no additional enlargement of zone size. This indicates that at least 10 μL of ethanol should be used to ensure maximum cycloheximide desorption and to minimize errors caused by accidental variance in the amount of ethanol applied. Controls consisting of resin beads equilibrated in aqueous and broth solutions of zero cycloheximide concentration consistently produced no inhibition zones.

Figure 2 shows the linear relationship between logarithmic concentration and the squared inhibition zone radius for three sets of data. The sets are those in which one, three, and five XAD-8 beads were placed in each well. This linearity is consistent with the basic theory of antibiotic inhibition zones.[5]

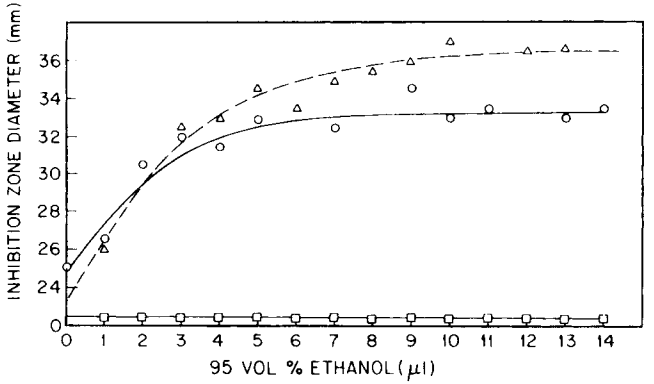

Fig. 1. Inhibition zone diameters as a function of the volume of ethanol (95 vol %) added to the wells: (△) XAD-4 with 50 μg/mL cycloheximide standard, (○) XAD-8 with 50 μg/mL cycloheximide standard, (□) ethanol only.

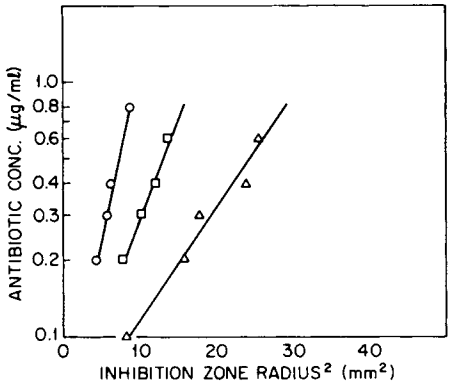

Fig. 2. Antibiotic concentration (cycloheximide) as a function of the inhibition zone radius^2 of an XAD-8 resin based microbiological assay: (○) one, (□) three, or (△) five resin beads were placed in each well of the agar plate.

The difference that arises between the case of resin beads eluted with ethanol and the traditional placement of liquid in the agar well or the use of filter-paper disks is that the resin assay has taken advantage of the ability of polymeric resin to absorb and concentrate the cycloheximide from the solution and of ethanol to quickly elute it from the resin bead. Through this process a concentration of cycloheximide high enough to produce an inhibition zone can be achieved in the well, whereas the simple addition of the same amount of unconcentrated sample would produce no zone. This ability of resin beads to adsorb and concentrate trace quantities has previously proven useful in assays by gas chromatography and mass spectroscopy.[6] The use of three and five beads per well is an effective method of raising the concentration in the well even further and thus lowering the minimum concentration detected.

It should be noted that the direct addition of ethanol to the resin bead is a modification of the technique originally developed.[1] It has two important advantages over the previous method of incorporating ethanol into the agar media. The first is that the inhibition zone sizes for any given cycloheximide concentration are larger than before. The second is that ethanol added in this manner has no inhibitory effect on the yeast growth. Thus, zones have developed and can be read after 24 h rather than 48 h.

Figure 3 shows the results obtained when this technique is extended from the simple two-component system of cycloheximide and water to that of a multicomponent system that might be encountered in an actual environment. Graphed together for comparison are the results of XAD-8 in the two-component system and in the fermentation broth and supernatant systems described earlier. Also shown are the results obtained with XAD-7 and aqueous standards. It can be seen that the linear relationship is not disrupted by the addition of other organic components of varying concentration. The lines obtained for XAD-8 with supernatant and broth are identical but they

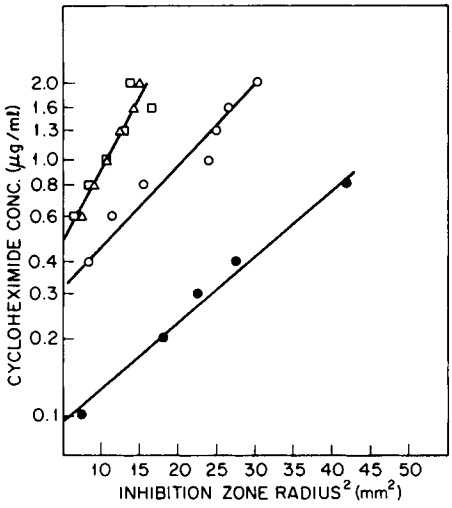

Fig. 3. Comparison of the cycloheximide concentration as a function of inhibition zone radius^2 for (□) broth, (○) standard, and (△) supernatant solutions, all using XAD-8 resin. Also shown for comparison are some results obtained with XAD-7 resin (●).

are separate from the line of XAD-8 with aqueous standards. For any given concentration in the range tested, the zone size resulting from broth or supernatant is always smaller than that for the aqueous standards. We believe that competition for adsorbance by one or more of the other organic components is responsible for this shifting of the line.

The usefulness of this assay is depenent on the ability to change resin bead characteristics with regard to the specific assay. Figure 3 shows the results of an attempt to make the assay more sensitive to low concentrations by increasing the surface area of the bead. The XAD-7 resin used has a surface area of 450 m^2/g vs. 140 m^2/g for the XAD-8, but has the same chemical composition.[1] The lowest concentrations detected with XAD-7 were about three to four times lower than that detectable by XAD-8. An additional advantage of XAD-7 resin over XAD-8 was the uniformity of zone shape. Irregular zone shapes had occurred at the lower end of the concentrations assayed with XAD-8. These irregularities occurred at an even lower concentration when XAD-7 was used. These irregularities can also be corrected by using multiple beads as demonstrated in Figure 2.

Figure 4 shows the results of applying the resin-based microbiological assay to the higher range of concentrations needed to monitor the model cycloheximide fermentation system. This graph of logarithmic cycloheximide concentration versus squared zone radius was obtained by using 5-mL volumes of whole broth, supernatant, and aqueous standards in conjunction with the XAD-8 resin-based assay. The data represented by Figure 4 were collected before all of the factors that affect the assay response had been standardized. Although this resulted in a somewhat high degree of variability,

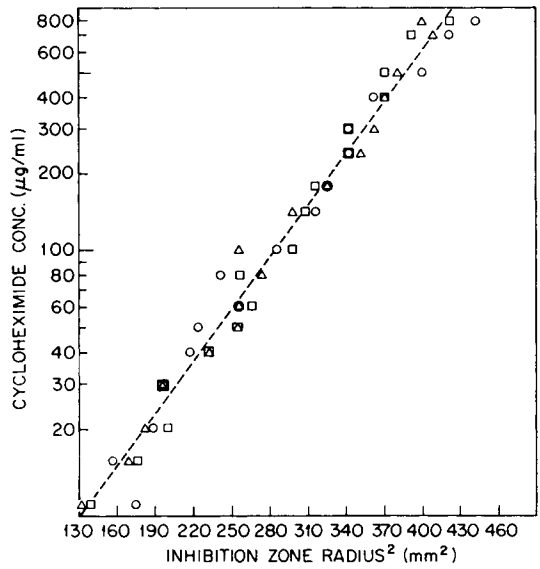

Fig. 4. Antibiotic concentration as a function of inhibition zone radius^2 for XAD-8 resin assay. (□) Supernatant and (△) whole broth samples were taken from a *Streptomyces griseus* fermentation; (○) aqueous standard.

Figure 4 still illustrates some of the characteristics and abilities of the resin-based assay. Here the linear relationship extends over approximately two orders of magnitude, from 10 to 800 μg/mL. Moreover, the coincidence of the aqueous, broth, and supernatant lines indicates that this relationship is not disrupted by the presence of media constituents, cell by-products, or the cells themselves at higher product concentrations. A similar relationship was obtained with the use of XAD-4 resin. This indicates that direct application of this assay to the determination of cycloheximide concentrations in fermentation broth would require no dilution or centrifugation of samples, whereas the normal paper-disk assay, applicable over a smaller range of concentrations, requires varying amounts of dilution, depending on the expected antibiotic concentration. Another advantage of the resin-based assay is that the small size of the resin bead as compared to the filter-paper disk allows for a more efficient use of a given area of agar.[1] This could be an important factor in lowering assay costs.

After standardizing the assay procedure the standard deviation present in the zone size produced by XAD-8 resin beads equilibrated with 50 μg/mL of cycloheximide, as determined from ten simultaneous trials, was ±0.530 mm. This corresponded to an error in concentration determination of approximately 13%. We feel a large part of this error can be traced to the method in which our experiments were run. Since each inhibition zone was formed in individual petri dishes, the uniform agar depth needed to ensure accuracy was not present. Also, since only eight dishes were made from each

flask of inoculated agar, the amount of inoculum present in any dish may not have matched the others in the group. The error that arises from these two sources is conceivably correctable in the industrial setting where large uniform sheets of agar are readily available. Some indications that this is possible are detectable by examining the correlation coefficient of data used in a linear regression to determine the straight-line relationship between the logarithm of concentration and squared zone radius. One such coefficient determined from 18 points taken from 18 petri dishes was 0.973, while the coefficient for a line determined from four points derived from four inhibition zones on the same plate was closer to a perfect fit at 0.998.

Application of Assay to Fermentation Monitoring

Very encouraging results, in terms of assay performance, were obtained when we monitored small screening vial fermentations with the resin-based assay. Figure 5 shows a small vial fermentation profile of cycloheximide concentration versus time. Each point represents a vial that was sampled at the given time by one of three methods. The three methods were the chemical assay, the equilibration of an XAD-8 bead for two hours followed by the resin-based assay, and the use of an XAD-8 resin bead that had been in the vial from time zero. Since the density of XAD-8 resin is close to that of the fermentation broth, the possibility of appreciable shear forces in the eddies, caused by the high rotation speed of the vials, is negligible. As can be seen from Figure 5, there is a good match between the profiles given by each of the three methods. The ability of the resin-based assay to determine the cycloheximide concentration is equal to that of the more tedious chemical

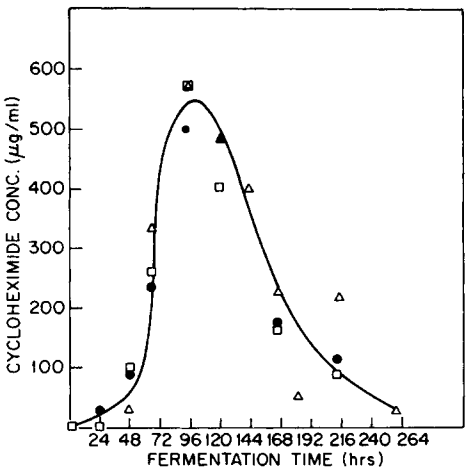

Fig. 5. Antibiotic production profile of small vial fermentations as determined by chemical and resin assay methods: (●) chemical assay, (□) resin assay (bead added for 2 h), (△) resin assay (bead added at $t = 0$).

assay procedure. Of the two methods of resin-bead addition shown, the convenience of adding the resin bead at time zero, along with the broth, makes it the clear choice over their repeated addition for two hours. The usefulness of this monitoring technique to industrial programs could be enhanced by the use of an automation scheme as described in the literature.[7]

As mentioned in the Introduction, the presence of rapid product degradation complicates the determination of peak product concentrations. But, by combining the resin-based-assay method in which the resin bead is added at time zero with a bead from which cycloheximide does not easily desorb, this problem may conceivably be overcome. We predict that an assay run in this manner will yield inhibition zones corresponding to that peak concentration regardless of the amount of degradation that has occurred in the broth. Any screening program run under these conditions would require only a single assay to determine which mutant has produced the highest peak product concentration. The heavy costs incurred by the determination of complete profiles could then be avoided.

An experiment identical to that represented in Figure 5 was run using XAD-4, a resin with a stronger affinity for cycloheximide than XAD-8, in place of XAD-8. In this case, the XAD-4 resin assay still indicated a drop in the antibiotic concentration following its peak but this drop was slower than that indicated by the chemical assay. This behavior supports our prediction that a resin may be found that will enable determination of the maximum concentration at any time after it is reached. We are currently working towards this goal.

The authors acknowledge S. Kirkwood for his technical assistance and Mrs. Dian Bennett for typing the manuscript. A grant from NSF (CPE-8010868) made this work possible.

References

1. H. Y. Wang, *Dev. Ind. Microbiol.*, **22,** 739 (1981).
2. L. A. Kominek, *Antimicrobiol. Agents Chemother.*, **7,** 856 (1975).
3. M. Takeshita, H. Takahashi, and T. O Kuda, *Chem. Pharm. Bull.*, **10,** 304 (1962).
4. A. J. Whiffen, *J. Bacteriol.*, **56,** 283 (1948).
5. W. Hewitt, *Microbiological Bioassay* (Academic, New York, 1977).
6. J. S. Fritz, *Ind. Eng. Chem.*, **14,** 94 (1975).
7. J. Geigert, P. Hansen, C. McDowell, R. Merrill, and C. Ward, *Dev. Ind. Microbiol.*, **17,** 153 (1976).

Accepted for Publication July 13, 1982