ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, June 1975, p. 861-863

Vol. 7, No. 6
Printed in U.S.A.

Cycloheximide Production by *Streptomyces griseus*: Alleviation of End-Product Inhibition by Dialysis-Extraction Fermentation

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Received for publication 5 March 1975

The use of dialysis fermentation with the continuous extraction of the dialysate has resulted in a twofold increase in the cycloheximide titer due to relief from product inhibition. Continuous extraction of the dialysate has eliminated the necessity for large reservoir volumes of fermentation medium normally used in dialysis fermentation. The apparatus used in this procedure is described.

Previous studies have shown that cycloheximide interferes with its own production, possibly due to feedback inhibition, and that cycloheximide is rapidly degraded in the absence of glucose (6). The problem of cycloheximide degradation was minimized by maintaining an excess of glucose in the medium with a sugar feed. The physical removal of cycloheximide during its production was investigated as a means for alleviating product inhibition by this antibiotic.

The usefulness of dialysis fermentation for the elimination of toxic products or the propagation of high cell concentrations has been described in the literature (1-5). A major obstacle in the economical employment of such a system for relieving product inhibiton is the large reservoir volume of medium that are required. This paper deals with the use of dialysis fermentation with continuous extraction of the dialysate which eliminates the need for a medium reservoir. The use of this apparatus and procedure has successfully relieved product inhibition in the cycloheximide fermentation.

MATERIALS AND METHODS

Microorganism cultivation. *Streptomyces griseus* was the organism used throughout these studies. The components used for agar slant, seed, and fermentation media are the same as described previously (6). The fermentations were run in a fermentor (New Brunswick Scientific Co., model MF 105) that contained 3 liters of fermentation medium. The fermentation medium was inoculated with 5% seed culture and run at 25 C. The aeration rate was 1.5 standard liters per min and the agitation was maintained at 700 rpm. An incremental glucose feed was initiated on day 2 of the fermentation with 60 ml of a sterile glucose solution (600 g/liter) added per day. Therefore, starting on day 2 of the fermentation, the medium was supplemented with 12 mg of glucose per ml every 24 h.

Procedure and apparatus for continuous dialysis-extraction. Figure 1 is an illustration of the apparatus constructed for dialysis extraction. A rubber mesh mat was fastened around the baffles of the fermentor (A) as a support for the dialysis tubing. Cellulose dialysis tubing (Union Carbide Corp., size identity no. 8) was used. The total dialysis surface area exposed to the fermentation medium was $\simeq$500 cm^2. The dialysis tubing in the fermentor was sterilized in place along with the fermentation medium by autoclaving. No contamination problems in the fermentor were observed. The dialyzing fluid was pumped to the fermentor and passed upward through the tubing at a flow rate of 40 ml per min.

The dialysate then entered the extractor (B) through glass tubing, which was constricted at the exit point to produce fine globules. The extractor consisted of a 1-liter graduate cylinder to which 900 ml of methylene chloride ($MeCl_2$) and 100 ml of water were added. The fluids were stirred with a magnetic stirrer and the cycloheximide was extracted from the dialysate during its passage through the $MeCl_2$ phase into the aqueous phase. The extraction was quite efficient and no cycloheximide was detectable in the aqueous phase of the extractor.

$MeCl_2$ which has been solubilized in the dialysis fluid is removed by aeration of the aqueous phase in the extractor. The water reservoir (C) was needed to replace fluid lost to the system by evaporation of the $MeCl_2$. Water addition was controlled by a probe that turned on a pump when the liquid level in the extractor dropped below a minimum level. The water reservoir could be replaced by a solvent reservoir if a means of maintaining a constant level of solvent in the extractor was developed. Another alternative would be to condense the $MeCl_2$ evaporated from the aqueous phase of the extractor and return it to the solvent phase of the extraction vessel. The $MeCl_2$ phase was changed daily in the operation of these experiments, but in practical operation the rate of fresh $MeCl_2$ required would be determined by its rate of loss from the extraction through solubilization in the dialyzing

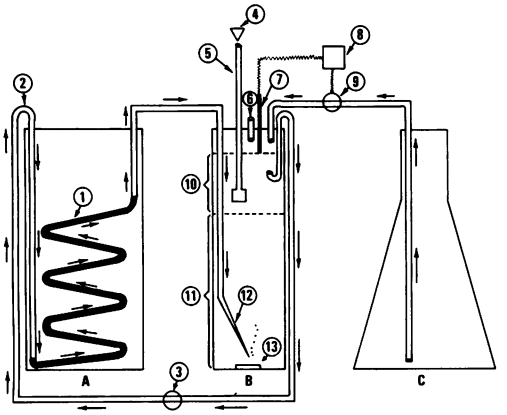

FIG. 1. *Apparatus for the continuous dialysis-extraction of the cycloheximide fermentation. The vessels used were: (A) fermentor, (B) extractor, (C) water reservoir. Other equipment used includes: (1) dialysis tubing, (2) rubber tubing, (3) peristaltic pump at 40 ml/min, (4) air supply (3 standard liters per min), (5) sintered glass sparger, (6) air outlet, (7) constant level probe, (8) relay system for probe, (9) peristaltic pump, (10) aqueous layer of water, (11) solvent layer of $MeCl_2$, (12) glass tubing, (13) magnetic stirring bar.*

fluid and by the maximal concentration of cycloheximide in the $MeCl_2$ phase which would still permit efficient extraction of the dialysate.

Sampling and assay. The fermentor and the solvent phase of the extractor were sampled daily. Cycloheximide concentration was determined by two methods, microbiological assay and colorimetric assay. The procedures for both of these assays have been described previously (6). Samples from the solvent phase of the extractor were taken to dryness and the solids were redissolved in water prior to assay.

RESULTS

The production of cycloheximide by a standard fermentation and a fermentation with continuous dialysis-extraction is compared in Fig. 2. Identical incremental glucose feeds were initiated on day 2 of the fermentation in both cases. Dialysis-extraction was initiated at 2 days (47 h) and terminated on day 8 (194 h) of the fermentation. The titers expressed for dialysis-extraction fermentation are equivalent titers in that they include the cycloheximide removed from the fermentor by dialysis-extraction. The fermentor volume was maintained at 3 liters during the course of this fermentation by the incremental additions of the glucose feed.

In the standard fermentation, the rate of cycloheximide production began to drop off between day 3 and 4 when the titers reach 60% of their maximum. Maximum titer in this type of fermentation was achieved by day 7. In contrast, a fermentation run with dialysis-extraction resulted in cycloheximide production that was more linear with time. A 1.5-fold titer increase was achieved on day 7 and a twofold titer increase was achieved by day 10 when compared to the standard fermentation.

Figure 3 illustrates the amount of cycloheximide in the fermentor and the amount removed by dialysis to the extractor, expressed as the

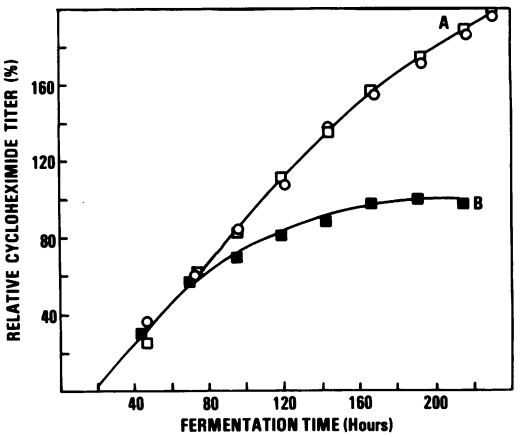

FIG. 2. *Comparison of cycloheximide production in a standard and a dialysis-extraction fermentation. (A) Glucose-fed fermentation with continuous dialysis-extraction. Cycloheximide was determined by chemical assay (○) and by microbiological assay (□). (B) Standard fermentation with a glucose feed. Cycloheximide was determined by the microbiological assay (■).*

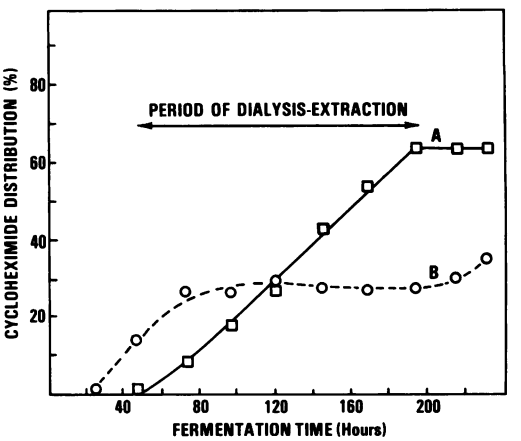

FIG. 3. *Accumulation of cycloheximide in the fermentor and extractor during a continuous dialysis-extraction fermentation. Cycloheximide was determined by the microbiological assay. (A) Percentage of total cycloheximide produced that was removed by dialysis to the extractor. (B) Percentage of total cycloheximide produced that remained in the fermentor.*

percentage of the total amount of cycloheximide (fermentor and extractor) accumulated at the end of the fermentation. Initially the rate of cycloheximide synthesis exceeds the rate of its removal by dialysis. The rate of cycloheximide biosynthesis by day 4 of the fermentation is sufficiently reduced by product inhibition that it equals the rate of removal by dialysis-extraction. If the cycloheximide titer in the fermentor could be maintained at a still lower concentration by optimization of the dialysis-extraction procedure, faster rates of antibiotic synthesis and subsequently still higher titers are probable.

Portions of the $MeCl_2$ used for dialysis-extraction were dried to a constant weight and the total solids present in this solvent were determined on a daily basis. On the average, cycloheximide accounted for 82% of the solids present in the solvent phase of the extractor, illustrating the selectivity of the process for this antibiotic.

DISCUSSION

The use of continuous dialysis-extraction with the cycloheximide fermentation has resulted in a twofold titer increase. Removal of cycloheximide by this process results in relief from end-product inhibition, allowing increased rates of cycloheximide synthesis to occur.

A major disadvantage to dialysis fermentations described in the literature was that they required large reservoir volumes of fermentation medium. In some cases the reservoir to fermentor relationship was 10:1. Incorporating extraction of the dialysate into this process has eliminated the need for these large reservoirs. The process of dialysis-extraction should be applicable to any fermentation product that is dialyzable and possesses a favorable distribution in a suitable water immiscible solvent.

ACKNOWLEDGMENT

I wish to thank D. J. Swan for his assistance in performing these experiments.

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