Appl Microbiol Biotechnol (2001) 56:384–387
DOI 10.1007/s002530100610

ORIGINAL PAPER

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Shear stress enhances microcin B17 production in a rotating wall bioreactor, but ethanol stress does not

Received: 5 September 2000 / Received revision: 4 December 2000 / Accepted: 8 December 2000 / Published online: 20 June 2001

Abstract Stress, including that caused by ethanol, has been shown to induce or promote secondary metabolism in a number of microbial systems. Rotating-wall bioreactors provide a low stress and simulated microgravity environment which, however, supports only poor production of microcin B17 by *Escherichia coli* ZK650, as compared to production in agitated flasks. We wondered whether the poor production is due to the low level of stress and whether increasing stress in the bioreactors would raise the amount of microcin B17 formed. We found that applying shear stress by addition of a single Teflon bead to a rotating wall bioreactor improved microcin B17 production. By contrast, addition of various concentrations of ethanol to such bioreactors (or to shaken flasks) failed to increase microcin B17 production. Ethanol stress merely decreased production and, at higher concentrations, inhibited growth. Interestingly, cells growing in the bioreactor were much more resistant to the growth-inhibitory and production-inhibitory effects of ethanol than cells growing in shaken flasks.

Introduction

Microcin B17 (MccB17) production by *Escherichia coli* ZK650 is controlled by the types and supply of sources of carbon, nitrogen, and phosphate, and by aeration (Connell et al. 1987; Fang and Demain 1997). Growth in rotating-wall bioreactors [RWBs; designed at the Johnson Space Center of the National Aeronautics and Space Administration to provide a low shear stress and simulated microgravity (SMG) environment] results in poor MccB17 production when conducted in the SMG mode (Fang et al. 1997a). Growth in agitated flasks or in the RWB operated in the normal gravity, high shear stress mode gave much better MccB17 production. Since stress has been reported to induce or increase secondary metabolism (see Discussion), we wondered whether high MccB17 production requires high stress. In the present work, we studied the application of shear stress and ethanol stress on growth and production of the antibiotic.

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Materials and methods

Media, strains, and fermentation conditions

The production culture was *E. coli* strain ZK650, an *E. coli* K12 derivative [ZK4 (F⁻*araD139* Δ*lacU169 rpsL relA thiA recA56*)+ pPY113] that contains the high-copy-number plasmid pPY113 (Yorgey et al. 1994). This plasmid was derived from pBR322 and contains *mcbABCDEFG* genes for ampicillin and tetracycline production plus genes coding for resistance to these antibiotics. The bioassay strain ZK4 (Yorgey et al. 1994) is the same as ZK650 but lacks the plasmid.

Stock cultures were prepared in Luria-Bertani (LB) broth containing Bacto-tryptone (Difco Laboratories, Detroit, Mich.), 10 g/l; Bacto-yeast extract (Difco), 5 g/l; and NaCl, 10 g/l. The medium (25 ml) was used in 250-ml unbaffled Erlenmeyer flasks. The cultures were shaken on a rotary shaker (2" diameter) at 200 rpm for 24 h at 37 °C. The cells were stored at –20 °C in 30% (v/v) glycerol as stock cell suspensions.

The seed medium was M63 plus glucose and ampicillin (Herrero and Moreno 1986). In g/l, it contained: glucose, 2; $(NH_4)_2SO_4$, 8; KH_2PO_4, 12; K_2HPO_4, 28; $MgSO_4 \cdot 7H_2O$; 0.25; thiamine, 0.001; and ampicillin, 0.15. The glucose was autoclaved separately from the rest of the medium. Thiamine was sterilized by filtration, and ampicillin was dissolved in 40% ethanol. Seed cultures of strain ZK650 were prepared by inoculating 1 ml of thawed stock cell suspension into 25 ml of medium M63 plus glucose and ampicillin in 250-ml unbaffled Erlenmeyer flasks. These seed cultures were incubated at 37 °C on the rotary shaker for 16–18 h. The production medium was the same except that glucose was used at 0.5 g/l. Flask fermentations were conducted in

250-ml unbaffled flasks (190 ml per flask), which were inoculated with 4% (v/v) seed culture and incubated on the shaker at 120 rpm at 37 °C for 48 h.

The RWBs (Synthecon, Houston, Tex.) have been described in previous papers (Fang et al. 1997b, c; Goodwin 1993). The bioreactors were positioned with the axis of rotation perpendicular to the gravity vector to produce SMG. The bioreactors were prepared and incubated for fermentation as previously described (Fang 1997c), except that 4% (v/v) of a seed culture was used.

Analyses

Samples were collected at 0, 16, 24, 40, and 48 h. Growth was determined by absorbance with the Klett Summerson colorimeter (Klett Manufacturing, New York, N.Y.) using the red filter. The fermentation broths were diluted with distilled water and the turbidity determined in the range of 50-150 Klett units. A dry cell weight (DCW) of 1 g/l is equivalent to 270 Klett units.

To assay MccB17, duplicate 0.5-ml samples of whole broth were placed in microcentrifuge tubes and centrifuged for 6 min. The supernatant fluids were put aside momentarily, 0.1 ml of 100 mM acetic acid containing 1 mM EDTA was added to each pellet, and the cells were extracted at 100 °C for 10 min. After cooling, the cell suspensions were added to their original supernatants, centrifuged for 6 min, and the resulting supernatant fluids used for bioassay. They were considered as 20% dilutions of the original broths.

The production of MccB17 was determined with the agar plate-disk diffusion assay using the assay strain ZK4 seeded at 100 µl of stock cell suspension per 100 ml of LB agar; the agar concentration was 8 g/l. Paper disks were saturated with 20 µl of sample and, after drying, were placed on the surface of the seeded agar. The bioassay plates were kept at 4 °C for 4–5 h to allow MccB17 to diffuse from the paper disks into the agar and then were incubated for 14-16 h at 37 °C. The diffusion step markedly increases the size of the clear inhibitory zones that result. After the 14–16-h incubation, diameters of the zones were measured and compared to a standard curve prepared with a semi-purified sample of MccB17. A solution containing 1 Unit per ml of activity produces a zone diameter equal to that given by a 1 µg per ml solution of that standard.

Results

Effect of addition of a bead to fermentations in RWBs

To create shear stress in the RWBs, a single Teflon bead (Teflon PTFE Solid Ball, 1/8-inch diameter; Norton Performance Plastic Corp., Wayne, N.J.) was added to 50 ml of medium and the fermentation was conducted in the SMG mode. The results were compared to those obtained with the RWB operated without the bead and with shaken flasks. Figure 1 shows that growth was stimulated slightly, and MccB17 production was considerably increased to a level over and above the effect on growth.

Effect of ethanol addition to flask fermentations

Unbaffled Erlenmeyer flasks of 250-ml capacity containing 190 ml medium and agitated at 120 rpm for 48 h at 37 °C constitute an excellent system for MccB17 production (Fang and Demain 1997). In order to evaluate the toxicity of ethanol to *E. coli* ZK650, we added various concentrations of ethanol from 0.5 to 5.0% to flask fermentations at 8 and 12 h. We found that MccB17 production is very sensitive to ethanol in that as little as 0.5% (v/v) markedly inhibited production. Growth was less sensitive to ethanol, showing inhibition only at 1.5% or higher concentrations. Ethanol at 5.0% totally inhibited growth and production (data not shown). Figure 2 shows the data obtained with 1, 2 and 3% ethanol added at 12 h, respectively. It can be seen that at no concentration did ethanol stimulate MccB17 production.

Effect of ethanol addition to fermentation in RWBs under SMG

A series of experiments was conducted in which one RWB was subjected to water addition at 12 h and another RWB received ethanol at concentrations ranging from 0.5 to 3.0%. Figure 3 shows the data obtained with 1, 2, and 3% ethanol. It can be seen that, in the RWB under SMG, growth was not inhibited by any of the concentrations of ethanol. MccB17 production was lowered only by the addition of 3% ethanol. Thus, as in flasks, MccB17 production was more sensitive to ethanol than was growth. It is interesting that cells grown in the RWB under SMG are much more resistant to ethanol than cells grown in flasks. A further experiment (data not shown) revealed that 5.0% ethanol added to the RWB culture totally inhibited growth, as it did in flasks. Of special importance is the observation that under no condition did ethanol stimulate production of MccB17.

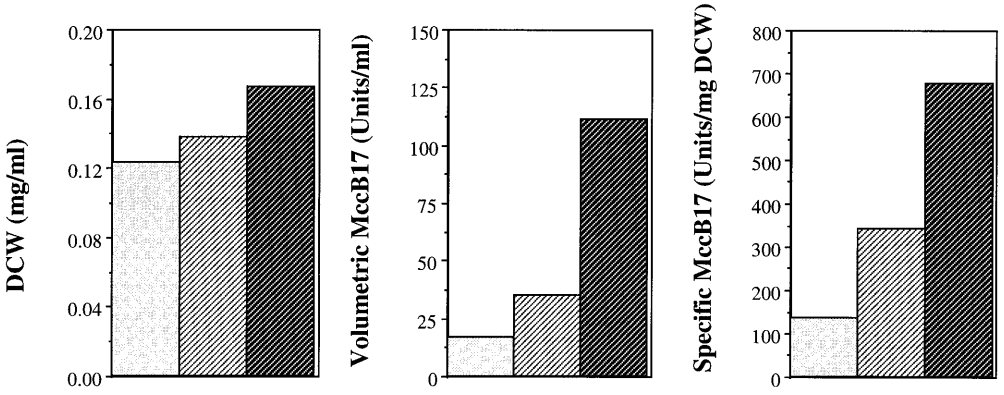

Fig. 1 Effect of one Teflon bead on growth (*left panel*), volumetric production of MccB17 (*middle panel*), and specific production of MccB17 (*right panel*) in rotating wall bioreactors. 0 Beads *gray-shaded columns*, 1 bead *hatched columns*. Also shown in each panel is the growth and MccB17 production in shaken flasks without a bead (*black-shaded columns*)

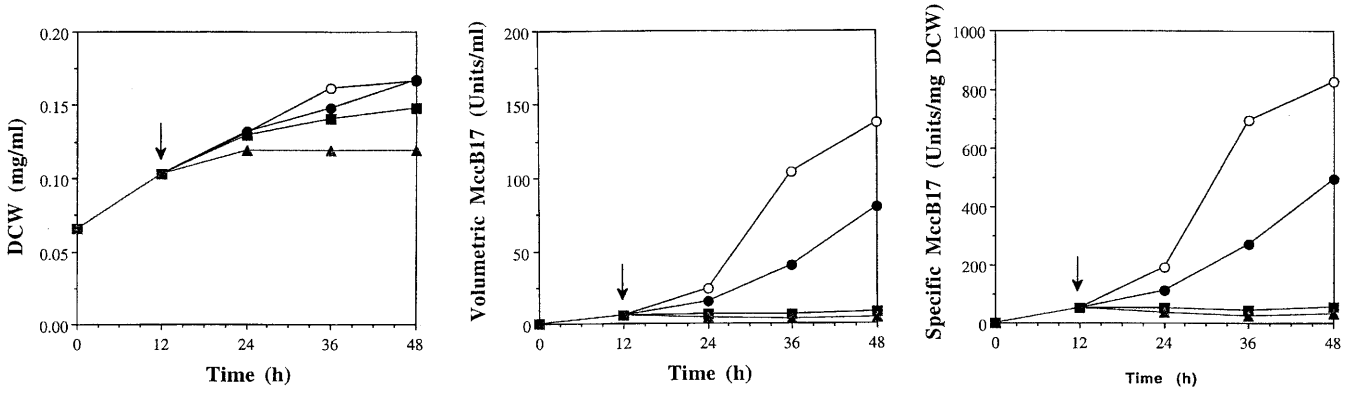

Fig. 2 Effect of ethanol on growth (*left panel*), volumetric production of MccB17 (*middle panel*), and specific production of MccB17 (*right panel*) in shaken flasks. *Arrow* Time of ethanol addition; ○ no ethanol, ● 1% ethanol, ■ 2% ethanol, ▲3% ethanol

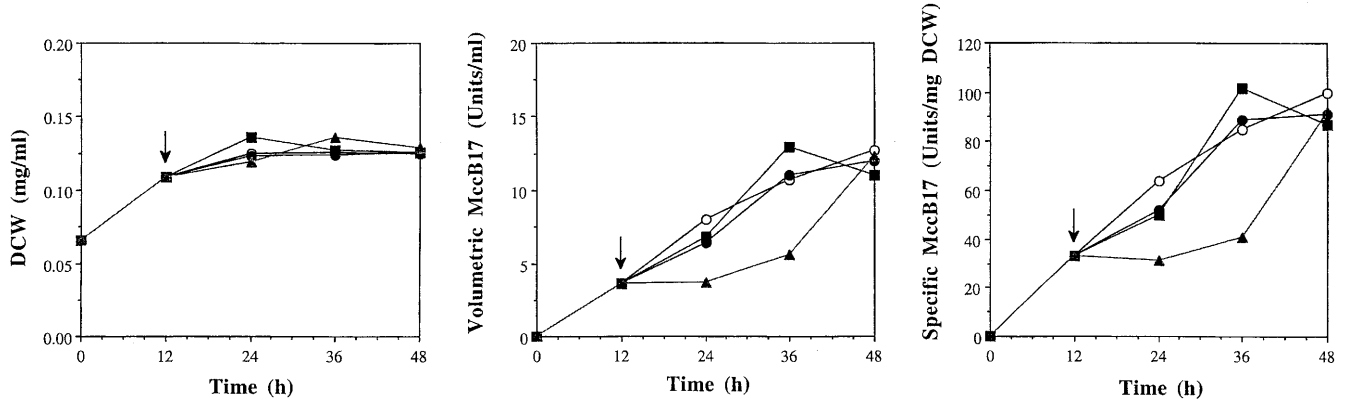

Fig. 3 Effect of ethanol on growth (*left panel*), volumetric production of MccB17 (*middle panel*), and specific production of MccB17 (*right panel*) in rotating wall bioreactors. *Arrow* Time of ethanol addition; ○ no ethanol, ● 1% ethanol, ■ 2% ethanol, ▲3% ethanol

Discussion

Much has been discovered on the effects of various types of stress on microbiological processes in recent years (Matin et al. 1999), and some work has been reported on the effects of stress on secondary metabolism, including the effect of ethanol. One would think that stress and secondary metabolism would be related, since the production of secondary metabolites is usually most active under conditions of nutrient limitation (Demain 1992), high oxygen transfer (Henriksen et al. 1997), and temperatures lower than that which is optimal for growth. Ethanol has been reported to induce or increase production of jadomycin by *Streptomyces venezuelae* (Doull et al. 1994), bacteriocins lactocin S and amylovorin L471 by lactobacilli (Mortvedt-Abildgaard et al. 1995; DeVuyst et al. 1996), antibiotics pyoluteorin and 2,4-diacetylphloroglucinol by *Pseudomonas fluorescens*, (Nakata et al. 1999), and bioconversion of penicillins to cephalosporins by *Streptomyces clavuligerus* (Fernandez et al. 1999). It also triggers a heat-shock response in *Staphylococcus aureus*, *Bacillus subtilis*, and *Salmonella typhimurium* (Lee et al. 1983; Arnosti et al. 1986; Qoron-Fleh et al. 1990). We thus wondered whether our previously reported examples of poor secondary metabolism in RWBs (Fang et al. 1997a, b, 2000a) were due to lack of stress in these reactors. In the present work, we found that shear stress does indeed increase production of a secondary metabolite such as MccB17, whereas ethanol stress is ineffective. This is not totally unexpected since there exists some degree of specificity in the types of stress proteins induced by different types of stress (Watson 1990; De Vuyst et al. 1996). An interesting observation made in this study was that growth and MccB17 production in the RWB are much more resistant to the negative effects of ethanol than are cells growing in shaken flasks. We had previously observed that such growth in RWBs is also much more resistant to carbon source repression (Fang et al. 2000b).

Acknowledgement This work was funded by NASA grant numbers NAG 9-602 and NAG 2-1350.

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