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Bacteriocin Production by *Clostridium acetobutylicum* in an Industrial Fermentation Process

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High titers of a noninducible bacteriocin were produced by *Clostridium acetobutylicum* in a molasses fermentation medium used for the industrial production of solvents. Release of the bacteriocin towards the end of the exponential growth phase was accompanied by lysis of the culture and inhibition of the production of solvents. The producer cells were sensitive to the bacteriocin, which only affected other *C. acetobutylicum* strains and a *Clostridium felsineum* strain. The thermolabile bacteriocin was not inactivated by protease enyzmes and had an optimum stability between pH 4 and 5. The sedimentation coefficient of the bacteriocin was 6S.

Clostridium acetobutylicum produces the solvents acetone and butanol from a variety of carbon sources in an industrial fermentation system, often referred to as the Weizmann process (17, 20). The microbial fermentation has been largely replaced by synthetic processes based on hydrocarbons, but as a result of the world energy shortage it is being reexamined by a number of laboratories. A factory fermentation plant producing acetone and butanol and using molasses as a substrate is still functional in South Africa. We describe the production by *C. acetobutylicum* of a bacteriocin, produced during the fermentation process, which could affect solvent yields.

Although bacteriocins of other *Clostridium* strains have been reported, this is the first report of a bacteriocin produced by *C. acetobutylicum*. The most extensively studied and characterized clostocins are those produced by pathogenic *Clostridium* strains (10-12, 21). The production of a bacteriocin-like substance by *Clostridium sporogenes* has been described (3), and four bacteriocins produced by nonpathogenic *Clostridium* strains have been characterized (8).

MATERIALS AND METHODS

Media. The potato medium used for the maintenance of the *C. acetobutylicum* strains and the assay of bacteriocin contained, in g per liter of distilled water: dried commercial potato flakes, 40; glucose, 6; $CaCO_3$, 2. The medium was mixed, autoclaved for 30 min, and filtered through cheesecloth, and the filtrate was reautoclaved for 20 min. The bacteriocin was produced in the fermentation medium, which consisted of (g): molasses, 134; $(NH_4)_2SO_4$, 2; $CaCO_3$, 1; and starch, 1, in 1 liter of distilled water. The pH of the medium was adjusted to pH 7 to 7.3. Agar media contained 1.5% (wt/vol) agar. Incubation was at 34°C, and plate cultures were incubated in GasPak (Becton, Dickinson & Co.) jars.

Bacterial strains. The *C. acetobutylicum* strains were supplied by National Chemical Products Ltd., Germiston, South Africa. The bacterial strains used in the activity spectrum studies were stock cultures from our department or supplied by P. Appelbaum, Microbiology Department, Medical School, University of Natal, Durban.

Bacteriocin assay. The bacteriocin was assayed by the well method (16) in 10-ml potato medium plates containing 1% (wt/vol) agar and seeded with *C. acetobutylicum* cells. The wells were filled with 25 μl of the test solutions. The indicator strain suspension in 0.85% (wt/vol) saline was prepared from an overnight plate of *C. acetobutylicum*. Bacteriocin titers in arbitrary units (AU) were expressed as the reciprocal of the highest doubling dilution that gave a zone of inhibition surrounding the well.

Bacteriocin production. Aliquots (0.4 ml) of exponential-phase cultures of *C. acetobutylicum* in molasses medium were inoculated into 200 ml of fresh molasses medium. Samples were removed at different time intervals and centrifuged at 10,000 rpm for 10 min at 4°C, and the supernatants were assayed for bacteriocin. The bacteriocin was precipitated by the addition of either $(NH_4)_2SO_4$ to 70% saturation or 50% (vol/vol) acetone at 4°C. The precipitate was collected by centrifugation at 10,000 rpm and resuspended in 0.05 M tris(hydroxymethyl)aminomethane-maleic acid buffer (pH 5.0).

Induction experiments. Mitomycin C (Calbiochem) and ultraviolet irradiation were used to test for induction of the bacteriocin. Exponential-phase cells were added to potato media containing 0.1, 0.5, 1.0, 1.5, and 3.0 μg of mitomycin C per ml and incubated for 2 h. The cells were then transferred to the molasses or potato media (18 ml), and the supernatants were assayed after 48 h. Induction by ultraviolet irradiation was carried out on exponential-phase cells

in potato medium which were diluted 10^{-2} in 0.85% (wt/vol) NaCl. Samples (7 ml) were irradiated in glass petri dishes with a Hanovia UV Lamp at a distance of 25 cm and a dose rate of 1.15 J/m^2·s for 30, 60, 90, and 120 s before adding to the molasses and potato media (15 ml) and incubating for 48 h.

Localization studies. Cell-bound bacteriocin was determined by washing the bacterial cells in 1 M NaCl and assaying the supernatant. Intracellular toxin was determined in the supernatants of sonically disrupted cell samples that had been clarified by centrifugation at 10,000 rpm.

Killing kinetics. Exponential cells were added to a bacteriocin solution, and the survivors were plated at different time intervals. The turbidity of the bacteriocin-treated cultures was also determined at the different time intervals.

Analysis of solvents. Aqueous samples of the molasses fermentation medium were injected into a Hewlett-Packard 5830A Chromatograph equipped with a flame ionization detector, and the quantitative evaluation of the peaks was performed automatically by the integrator using *n*-propanol as an internal standard. The stainless steel column was 1.84 m in length, with an internal diameter of 2 mm, and packed with Chromsorb W/AW (80-100 mesh) coated with 15% Carbowax 20M. Analysis of solvents was achieved under the following conditions: column temperature, 90°C (isothermal); injector temperature, 250°C; detector temperature, 250°C; N_2 (carrier gas) flow rate, 30 ml/min; H_2 flow rate, 40 ml/min; air flow rate, 500 ml/min. The retention times for acetone, ethanol, *n*-propanol, and butanol were 0.98, 1.40, 2.19, and 3.89 min, respectively.

Protease determinations. Protease activity at different time intervals throughout the 48-h fermentation in molasses medium was estimated by using the synthetic nonspecific protease substrate Azocoll (Calbiochem) (18), the hemoglobin method of Anson (1), and the caseinolytic paper-disk method of Uchino et al. (22).

RESULTS

Production of the bacteriocin. The production of bacteriocin by *C. acetobutylicum* strain P262 in the molasses fermentation medium is shown in Fig. 1. Low levels of bacteriocin were detected after 24 h. The concentration of bacteriocin increased rapidly between 27 and 40 h, and very high titers (4,000 AU) were routinely obtained. The increase in bacteriocin titer coin-

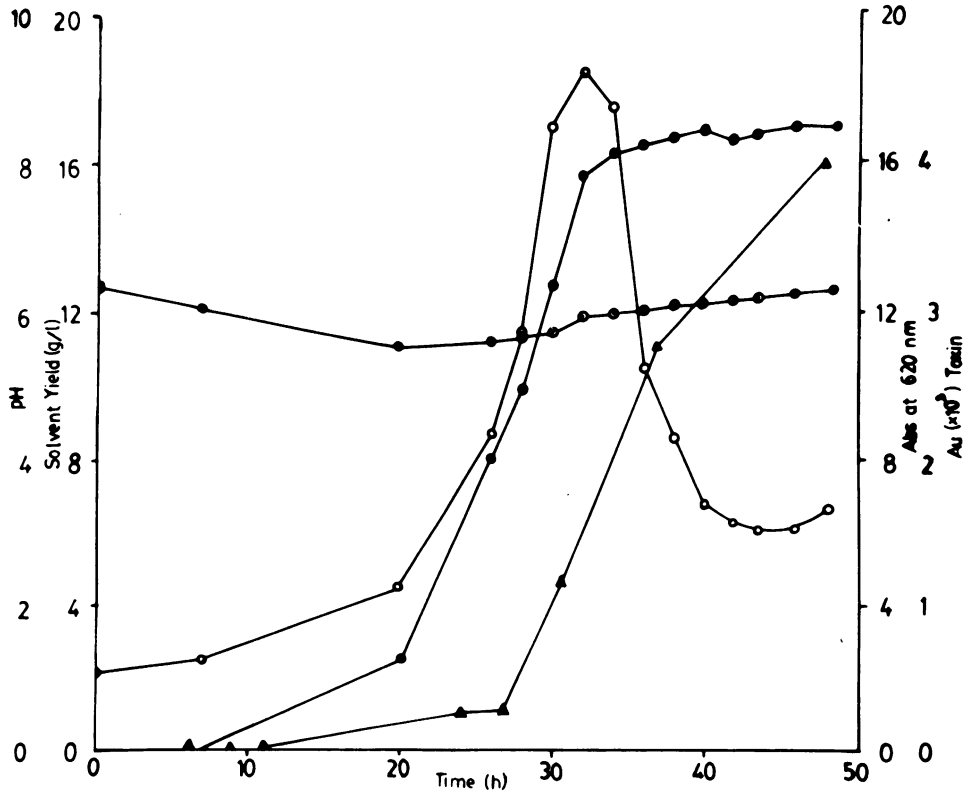

FIG. 1. *Detailed studies of pH changes, bacterial growth, and solvent and bacteriocin production during a C. acetobutylicum fermentation. Symbols:* (⊙) *pH;* (○) *turbidity;* (●) *total solvent production;* (▲) *bacteriocin production.*

cided with the end of the exponential growth phase and the marked decrease in turbidity of the bacterial culture. Mass lysis of the bacterial culture was not observed when low titers of the bacteriocin were produced. Induction with mitomycin C or ultraviolet irradiation did not result in increased bacteriocin titers. The total production of solvents (acetone, butanol, and ethanol) followed the bacterial growth curve, reaching a maximum of 16.9 g/liter after 40 h. Once the cells started to lyse there was no further increase in the solvent concentration, which plateaued at 34 h. The lysis of the cells was not caused by the solvents, since concentrations of 18.0 g/liter did not affect the growth of the *C. acetobutylicum* cells or produce zones of inhibition when tested in the well test. It is interesting that the rapid production of solvents coincided with what is known as the pH breakpoint, when the pH of the culture medium started to increase (Fig. 1).

Localization studies carried out at 48 h indicated that the majority of the toxin was extracellular (2,048 AU), compared to 512 AU intracellular and 32 AU cell bound.

Activity spectrum. The producer strain *C. acetobutylicum* P262 was sensitive to the bacteriocin that it produced when used as an indicator strain in the well test assay. An additional 11 *C. acetobutylicum* strains were also shown to be sensitive to the bacteriocin (Table 1). Furthermore, each of these strains produced and was sensitive to a similar bacteriocin, which also inhibited each of the other *C. acetobutylicum* strains. The bacteriocin was very specific, and the only other bacterial species that it inhibited was *Clostridium felsineum* (Table 1).

TABLE 1. *Activity spectrum of C. acetobutylicum bacteriocin*

Organism	No. of strains	
	Tested	Sensitive
Aerobes		
Achromobacter A_2	1	0
Escherichia coli	8	0
Serratia marcescens	1	0
Salmonella typhimurium	1	0
Bacillus subtilis	1	0
Anaerobes		
Bacteroides fragilis	1	0
C. acetobutylicum	12	12
C. perfringens	16	0
C. sporogenes	2	0
C. butyricum	1	0
C. felsineum	1	1
C. septicum	1	0
C. fallax	1	0

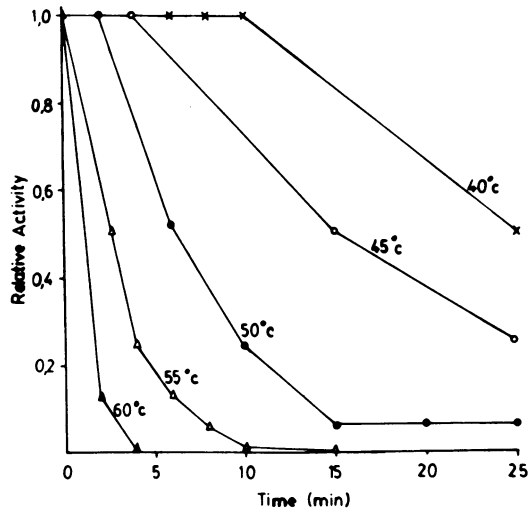

FIG. 2. *Thermal inactivation of the C. acetobutylicum bacteriocin.*

TABLE 2. *Effect of pH on the C. acetobutylicum bacteriocin*

pH	Toxin activity (AU)
4.0	16,384
4.4	16,384
5.0	16,384
5.4	8,192
5.8	8,192
6.1	8,192
6.5	4,092
6.7	4,092
7.1	4,092
7.5	4,092
8.0	2,048
9.0	512
10.0	64

Effects of physical and chemical agents and bacteriocin stability. The rate of thermal inactivation of the bacteriocin was determined by incubating crude samples of the bacteriocin at 40, 45, 50, 55, and 60°C for different time intervals. The bacteriocin was thermolabile and was totally inactivated after 4 min at 60°C ($t_{1/2}$ = 2 min at 60°C, Fig. 2). The bacteriocin was shown to be stable for 2 days at 0 to 4°C.

The optimum pH for the stability of the bacteriocin was determined by adding crude samples of the bacteriocin to buffers between pH 4 and 10 and assaying the activity after 30 h at 4°C. The optimum pH for the stability of the bacteriocin was between pH 4 and 5 (Table 2).

The proteolytic enzymes papain (Sigma), pepsin (Merck), Pronase (Miles-Seravac), trypsin (Difco), and chymotrypsinogen (Miles-Seravac),

and the nucleases deoxyribonuclease (Miles-Seravac) and ribonuclease (Miles-Seravac), at a concentration of 0.1 mg/ml did not inactivate the bacteriocin after incubation at 25°C for 2 h.

The bacteriocin was inactivated by 1% concentrations of the protein-denaturing agents sodium dodecyl sulfate (wt/vol) and phenol (vol/vol), but not by mercaptoethanol (vol/vol).

Crude samples of the bacteriocin were not inactivated by 1 mM concentrations of *N*-ethyl maleimide, *p*-hydroxy mercuribenzoate, dithiothreitol, ethylenediaminetetraacetic acid, phenanthroline, and cysteine hydrochloride. The bacteriocin was also not affected by chloroform.

The bacteriocin was retained by a dialysis membrane, but was not sedimented by centrifugation at 102,000 × *g* for 2 h. Negatively stained (2% [wt/vol] phosphotungstic acid) electron micrographs of the supernatant did not reveal any particles. The sedimentation coefficient of the bacteriocin was determined as 6S by the sucrose gradient method of Martin and Ames (15) using bovine catalase (Miles Laboratories) as a reference marker.

Killing kinetics. Addition of the bacteriocin to exponentially growing *C. acetobutylicum* cells caused a rapid decrease in viability. The killing effect did not reach a plateau, but continued, indicating that a resistant population was not selected (Fig. 3). The turbidity of the culture treated with bacteriocin remained constant over 60 min, indicating that the action of the bacteriocin is bactericidal but not bacteriolytic.

Protease activity. No protease activity was detected in high-titer bacteriocin solutions or at any stage during the molasses fermentation.

DISCUSSION

The production of high titers (2,000 to 4,000 AU) of bacteriocin by *C. acetobutylicum* in a molasses fermentation medium is not inducible, because the titers are not affected by inducing agents. The producer cells are sensitive to the lethal action of the bacteriocin. A lack of immunity has been reported for a few bacteriocinogenic strains (9, 19), and it is more prevalent among gram-positive bacteria than gram-negative bacteria (7). Since the action of the bacteriocin is bactericidal but not bacteriolytic, the lysis of the producer cells is assumed to be due to the liberation of the bacteriocin. Cultures that do not produce high titers of the bacteriocin do not undergo mass lysis. A *C. perfringens* bacteriocin has been found to cause spheroplast formation (13), but this did not occur on application of *C. acetobutylicum* bacteriocin.

Autolysis and toxin release has been reported in *C. botulinum* (4). Manfeitelj (14) described

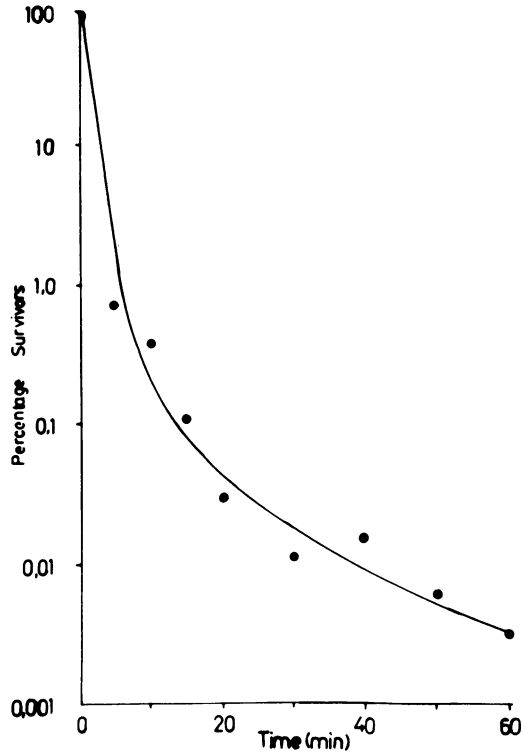

FIG. 3. *Kinetics of C. acetobutylicum bacteriocin-induced lethality.*

the production of proteases and autolysis in *C. acetobutylicum*. The mass lysis of the *C. acetobutylicum* strains described in this paper is not associated with the production of proteases.

The thermolabile nature and pH stability range of the *C. acetobutylicum* bacteriocin are similar to boticin E produced by *C. perfringens* (6). The *C. acetobutylicum* bacteriocin is also similar in some respects to a group of bacteriocins which are active against *C. acetobutylicum* and are produced by certain nonpathogenic *Clostridium* strains (8). These bacteriocins are thermolabile, are not induced by mitomycin C or ultraviolet irradiation, and have a wide pH stability range (pH 4 to 9) and a broad activity spectrum (8). The *C. acetobutylicum* bacteriocin is very specific, and only one other species, *C. felsineum*, is sensitive to the toxin. *C. felsineum* is closely related to *C. acetobutylicum* (5) and produces yields of acetone and butanol equivalent to one-half to three-quarters of that produced by *C. acetobutylicum* (2). The specific activity spectrum of the *C. acetobutylicum* bacteriocin could be useful in the identification of *C. acetobutylicum* strains.

The *C. acetobutylicum* bacteriocin was not inactivated by protease enzymes, but there are

indications that it is proteinaceous. It is inactivated by the protein-denaturing agents sodium dodecyl sulfate and phenol, is retained by dialysis membrane, has a sedimentation coefficient of 6S, and is precipitated by $(NH_4)_2SO_4$ and acetone. *Streptococcus faecium* also produces a bacteriocin, which is not affected by proteases but behaves like a protein (19). A number of other bacteriocins produced by gram-positive bacteria are only partially inactivated by proteases (7, 8).

The release of the bacteriocin by *C. acetobutylicum* is detrimental to the industrial production of solvents by fermentation. The rapid lysis of the culture towards the end of the exponential growth phase inhibits the further production of solvents. Prevention of bacteriocin production and lysis could result in an increase in growth and a normal growth curve with a concomitant increase in solvent yields.

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