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Controlling Morphological Instability of *Zymomonas mobilis* Strains in Continuous Culture

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Growth of *Zymomonas mobilis* ATCC 29191 and CP4 in a continuous stirred tank fermentor resulted in the selection of stable flocculating variants. Factors responsible for enhancing the system pressures selective for the morphological variants were identified. By incorporating some modifications into the design of the fermentor, it was possible to achieve steady-state operation of the chemostat with both wild-type and flocculating strains. Biochemical and microscopic studies were performed to elucidate the mechanism of flocculation in *Z. mobilis*.

Kinetic analysis of microbial fermentation in continuous culture is dependent on attaining good genotypic and phenotypic stabilities of the producer microorganism. This is essential to achieve steady-state operation of the fermentation over sufficiently long intervals so that proper sampling and measurement of kinetic parameters can be performed. Cultural stability is also a key consideration in the commercialization of continuous fermentation processes, since instability results in changes in process productivity and efficiency.

Over the past several years, we have been developing a novel industrial-scale fermentation process for the continuous production of fuel ethanol by *Zymomonas mobilis* (George Weston Ltd., U.S. patent pending [application serial no. 06/217,006]) (1a). During the course of this work, we have observed that different strains of this species tend to undergo spontaneous morphological change during growth in continuous culture, resulting in the appearance and accumulation of bacterial flocs in the fermentor. Floc formation is accompanied by a gradual buildup of biomass in the chemostat and a difficulty in achieving steady-state operating conditions.

Floc-forming variants of *Z. mobilis* have been isolated from nature (3, 21) and have been recently reported to arise spontaneously in immobilized-cell (1) and tower (16) fermentors and after chemical mutagenesis (19). Despite this, little is known about the physiology of the cells or the basis of flocculation. The use of flocculent variants of *Z. mobilis* as a means for increasing fermentor productivity is presently under consideration in this and other laboratories as an alternative to cell recycling by filtration (13, 17), cell immobilization (1, 9, 14), and the vacuum fermentation process (12). Flocculent yeasts are being employed in this capacity in the brewing industry (20).

Chemostats have long been known to be powerful devices for the selection of bacterial mutants (6, 10, 15) and other genetic variants (5, 11) and for enriching for particular types of microorganisms from nature (18). Theoretical considerations concerning strain selection in chemostats have been reviewed by Schlegel and Jannasch (18). In the present paper, factors favoring the selection of flocculent variants of *Z. mobilis* in the conventional continuous stirred tank fermentor are identified. By introducing small changes into the design of the system, we were able to control the morphological behavior of *Z. mobilis* in continuous culture, enabling kinetic studies to be carried out with both variant types under steady-state conditions. The results of the kinetic studies are reported elsewhere (8).

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MATERIALS AND METHODS

Microorganisms. The bacterial strains employed are listed in Table 1, together with their phenotypes and origins. The strains were lyophilized for long-term preservation.

Media. The cultures were grown anaerobically (GasPak Anaerobic System; BBL Microbiology Systems, Cockeysville, Md.) at 30°C on medium A, which contained (per 100 ml of deionized water) 1 g of yeast extract (Difco Laboratories, Detroit, Mich.), 2 to 5 g of D-glucose, 0.1 mg of biotin, and 0.1 mg of calcium pantothenate (filter sterilized; presterilization pH, 7.3). The medium was solidified with 2% (wt/vol) agar (Difco) and maintained at 4°C. Glucose and calcium

TABLE 1. *Z. mobilis* strains employed

Strain	Phenotype	Source
ATCC 29191[a]	WT[b]	American Type Culture Collection
CP4[c]	WT	O. Goncalve de Lima (21)
CP3	WT	O. Goncalve de Lima (21)
WR6	Flo[d]	Spontaneous mutant of ATCC 29191
WR4	Flo	Spontaneous mutant of CP4
17.3	Flo	Natural flocculating strain (21)

[a] Alternate designations: Z6 (21), NCIB 11199, and NRRL B-4490.
[b] WT, Wild type.
[c] Alternate designation: Zm4 (19).
[d] Flo, Flocculation; forming visible granular deposits in liquid culture.

pantothenate were prepared separately and added to the other medium components after being sterilized.

Two media, designated B and C, were used in the continuous culture experiments. Medium B contained the following (grams/100 ml of deionized water): yeast extract, 1 (adjusted to pH 4.5 with HCl before sterilization); NH_4Cl, 0.16; $MgSO_4 \cdot 7H_2O$, 1; KH_2PO_4, 0.25; biotin, 10^{-4}; calcium pantothenate, 1.5×10^{-4}; polypropylene glycol 2025 antifoam (0.1% [vol/vol]), and D-glucose (analytical grade), 10 to 13. The medium was prepared by autoclaving the glucose plus polypropylene glycol 2025, salts plus biotin, and yeast extract as separate solutions and then mixing them together after cooling. Calcium pantothenate was filter sterilized and added last. Medium C (7) was the same as medium B except that it contained yeast extract at 15 g/liter and no added salts. The pH values of both media in the chemostat were controlled at 5.5 with 2 N KOH.

Continuous culture. Cells from a single colony grown on solidified medium A were transferred to 100 ml of liquid medium A in a 125-ml flask and incubated for 24 to 48 h at 30°C without agitation. Ten milliliters of this culture was used as the seed for inoculating chemostats (BioFlo model C30 and Multigen model F2000; New Brunswick Scientific Co., Edison, N.J.) containing medium B or C. The approximate operating volumes used in these fermentors were 0.37 and 1 liter, respectively. Unless otherwise indicated, culture volumes in the chemostats were maintained constant by an overflow weir (model C30) or siphon (model F2000). The cultures were grown overnight at 30°C under batch conditions before initiating nutrient feed flow. The temperature and pH were controlled at 30°C and 5.5, respectively. Nitrogen gas was bubbled slowly through the cultures to maintain anaerobic conditions and help drive off the dissolved carbon dioxide produced as a fermentation by-product. Agitation rates are expressed as impeller tip velocity (meters per second), which is related to revolutions per minute by the formula: impeller tip velocity = (revolutions per minute/60) $\cdot$ (πD), where D is the impeller diameter (4.91×10^{-2} m).

To establish steady-state operating conditions, at least three volume changes of culture medium were passed through the fermentor before sampling at each new dilution rate (D). Thereafter, samples were taken at approximately 24-h intervals until steady-state conditions were verified on the basis of biomass, substrate, and product concentrations.

Isolation of stable flocculating mutants. Stable spontaneous flocculent mutants were isolated from continuous cultures of *Z. mobilis* ATCC 29191 and CP4. Large bacterial clumps present in 20-ml samples of broth collected asceptically from the fermentors were allowed to settle out of suspension (10 s). After decanting the liquid fraction containing small flocs and nonflocculated cells, the sedimented clumps were gently resuspended in 20 ml of medium A and then allowed to resettle. Ten cycles of floc enrichment were performed, after which the clumps were partially disrupted with a Pasteur pipette. The bacteria were then plated and purified by cross-streaking twice on solid medium A. The isolates were tested for stability of the flocculence phenotype by serially transferring the cells to static broth tubes containing 10-ml portions of medium A. Unstable mutants and the wild-type strain produced a hazy growth throughout the medium. The stable flocculent strains, on the other hand, grew as heavy deposits at the base of the tubes, with the overlying broth remaining transparent.

Bottom-emptying level control device. To prevent selection of flocculent variants, we modified the model F2000 fermentor to allow withdrawal of the effluent from the base of the vessel (Fig. 1). A level control device was designed on the basis of the ability of a thermister probe (5 mA) to respond to slight temperature differences between the gaseous phase and the liquid phase in the fermentor. A change in temperature causes a change in resistance in the thermister. The voltage change in the circuitry is then amplified, causing the relay to activate or deactivate a solenoid valve or pump.

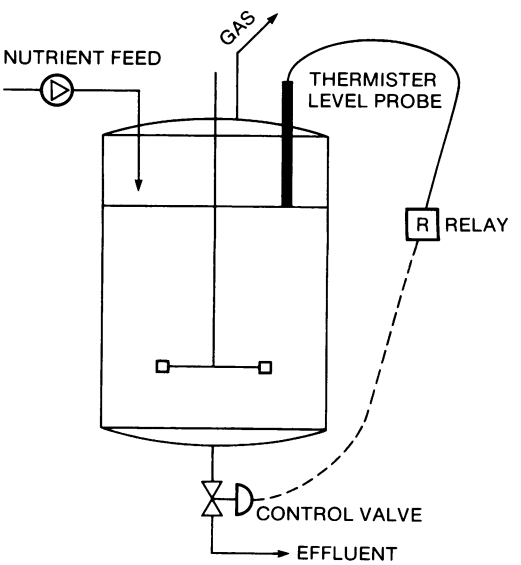

FIG. 1. Schematic diagram of the chemostat vessel with a bottom-emptying level control device.

Analytical procedures and biomass determination. Glucose concentrations were measured by high-pressure liquid chromatography with an HPX87H column (Bio-Rad Laboratories, Richmond, Calif.) and 0.01 N H_2SO_4 as the eluent (1.0 ml/min). Detection was based on refractive index (differential refractometer, model R401; Waters Associates, Milford, Mass.). Data integration was performed with a Spectra Physics 4000 data processor. The retention time and minimal level of detection for glucose were 0.50 min and 0.01% (wt/vol), respectively.

To minimize fermentation activity after sampling, especially in samples containing high biomass concentrations, the chemostat samples were collected into prechilled vials on ice and then immediately spun for 15 s at 15,600 × *g* in an Eppendorf model 5414 microcentrifuge. The supernatant solutions were saved for glucose analysis. For biomass determinations, cells from duplicate 5-ml samples were collected at 4°C by centrifugation (8,000 × *g*, 5 min) in a model RC2B centrifuge (Ivan Sorvall, Inc., Norwalk, Conn.), washed once in cold deionized water, suspended in cold deionized water, transferred to preweighed weighing boats, and weighed after 24 and 48 h of drying at 60°C. To ensure an even distribution of flocs in the fermentor at the time of sampling, the impeller tip velocity was increased to 0.77 m/s. After the sample was obtained, the impeller tip velocity was readjusted to the original agitation rate.

Scanning electron microscopy. Samples of cells in chemostat medium were fixed with Formalin (7% [wt/vol]), dehydrated in a graded series of ethanol, and critical point dried. The bacteria were mounted on double-sided tape and sputter coated for 3 min (0.2 to 20 nm) with gold-palladium (60:40). They were then viewed with an Etec Autoscan scanning electron microscope at 10 and 20 kV.

RESULTS AND DISCUSSION

Selection of spontaneous flocculating variants. During continuous fermentations in a model C30 chemostat with a conventional overflow weir, *Z. mobilis* ATCC 29191 and CP4 frequently underwent spontaneous morphological change resulting in floc formation. In a typical experiment with CP4 in medium B containing 12% glucose, flocs were first detected 29 h after switching to the continuous culture mode of operation (D, 0.09 h^{-1}; impeller tip velocity, 0.77 m/s). The *D* was thereafter increased in gradual steps, and by day 12, at a D of 0.46 h^{-1}, the culture showed extensive flocculation that was clearly visible to the naked eye. From this experiment, the stable flocculent mutant designated WR4 was isolated. Similar results were obtained with ATCC 29191 in chemostats containing glucose or sucrose as the carbon source. ATCC 29191, however, appeared to be considerably more stable than CP4. In the experiment giving rise to mutant WR6, bacterial clumps were first observed during the day 9 of continuous culture in a model C30

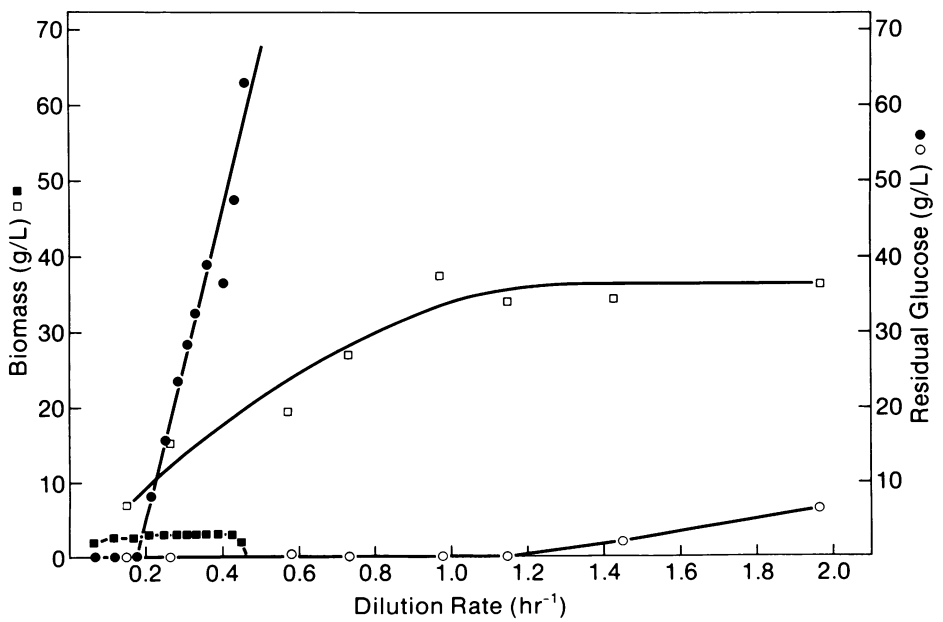

FIG. 2. Comparative growth of strain ATCC 29191 and its isogenic flocculent variant WR6 in a model C30 chemostat. The cells were cultured at different D values in medium C containing 10% D-glucose. Agitation was set at the lowest rate capable of preventing the accumulation of biomass on the bottom of the fermentor (approximate impeller tip velocity, 0.13 m/s). Symbols: ■, ATCC 29191 biomass concentration; □, WR6 biomass concentration; ●, residual glucose concentration in ATCC 29191 culture; ○, residual glucose concentration in WR6 culture.

fermentor with medium B containing 10% D-glucose (D, 0.26 h^{-1}; impeller tip velocity, 0.32 m/s). As did WR4, WR6 exhibited a stable flocculence phenotype during serial subcultures in static broth.

Skotnicki et al. (19) have recently reported that strain CP4 (referred to by Skotnicki et al. as ZM4) will not flocculate spontaneously and that the isolation of such variants (e.g., ZM401) requires chemical mutagenesis. It is our understanding that this mutant is the subject of a patent application (P. L. Rogers, personal communication). Contrary to their findings, we found that CP4 quite readily underwent spontaneous mutation, resulting in the formation of stable, highly flocculent variants. In static broth culture, small CP4 flocs could usually be seen after 24 to 48 h of incubation. The flocculent bacteria could be enriched and isolated by serial static broth transfer as described above. Another strain, CP3, was also found to flocculate readily in static broth culture, in contrast to ATCC 29191.

The two stable flocculent mutants, WR4 and WR6, were used to ascertain the basis of flocculent variant selection in the fermentor. When grown in medium B or C in continuous culture (model C30 or F2000) with low agitation (impeller tip velocity, 0.77 m/s) and a D of 0.9 h^{-1}, the mutants exhibited heterogeneous distribution, with the lowest visible biomass concentrations occurring nearest the surface of the broth (i.e., in the vicinity of the overflow port). This provided a logical explanation for the selection of flocculent variants in the vessel, since heterogeneously distributed flocs are much less likely to be washed out of the fermentor than homogenously distributed wild-type cells. Thus, in a fermentor with an overflow weir (available from New Brunswick Scientific Co.), the wild-type strain would be at a selective disadvantage.

Support for this theory was obtained by growing strain WR6 in a modified model F2000 chemostat having a bottom-emptying weir, with the D and impeller tip velocity maintained at approximately 0.28 h^{-1} and 0.05 m/s, respectively. Under these conditions, the clumped bacteria would be expected to be at a selective disadvantage, compared with nonflocculent cells. Such a system indeed was found to favor selection of spontaneous revertants of WR6. Over the course of fermentation (12 days), the culture gradually changed in appearance from a transparent broth with large suspended clumps to a turbid suspension characteristic of nonflocculent bacteria. To determine the approximate proportion of stable revertants in the fermentor at day 12, the effluent line was clamped off, and the agitation was increased for 1 min to an impeller tip velocity of 2 m/s, thus dissociating any remaining clumped bacteria. After plating for single colonies on solid medium A, none of 50 randomly picked isolates was found to be flocculent when tested by serial static broth subculture, indicating that the revertant cells accounted for >98% of the population in the fermentor. In contrast, of 50 WR6 colonies tested in a similar manner as controls, only one produced any nonflocculent growth. The development of this modified chemostat has enabled us to investigate the kinetics of fermentation with nonflocculent strains of *Z. mobilis* and to perform long-term continuous fermentations under conditions of culture stability (8).

In addition to the position and design of the effluent weir, several other factors were found to influence the selection of flocculent variants in the chemostat, i.e., D, agitation rate, and position and number of impellers, probes, and baffles. In both fermentor models, the WR4 and WR6 floc sizes and biomass concentrations increased significantly with D up to a D of ca. 1.0 h^{-1}, with little change thereafter (Fig. 2). The bacterial clumps were nonuniform in shape and had a maximum length of 2 to 4 mm. The increase in biomass concentration with D did not conform to traditional chemostat theory (10) and is probably attributable to an inability of the dense flocs to be washed out of the fermentor. In other words, the chemostat was functioning as if it had an internal cell recycling system. At high D values, the flocculent biomass was observed to rapidly settle out of solution when agitation was switched off (<1 min).

The biomass concentrations obtained in this system were much higher than that recently reported for a different flocculent *Z. mobilis* strain grown in a chemostat with cell recycling from an external settler device (3). The present system, with its "internal settler," is more feasible for commercialization, being much simpler, less costly, and less prone to contamination. Furthermore, it achieves significantly higher rates of ethanol production (8). Unlike the flocculent mutant, the wild-type strain, ATCC 29191, could not maintain itself in the fermentor at D values above 0.46 h^{-1}, a D value corresponding to the maximum specific growth rate of the strain in medium C (Fig. 2).

The effect of agitation rate on flocculent variant selection was demonstrated by the observed washout of flocculent strain WR4 from the model C30 chemostat operating at a D of 0.69 h^{-1} and a high (impeller tip velocity, 0.8 m/s) but not a low (impeller tip velocity, <0.18 m/s) agitation rate. In the latter case, a high steady-state biomass concentration of about 21.5 g/liter was achieved. Low agitation favors heterogeneous distribution of the dense cellular material and is less mechanically disruptive to the clumps. High

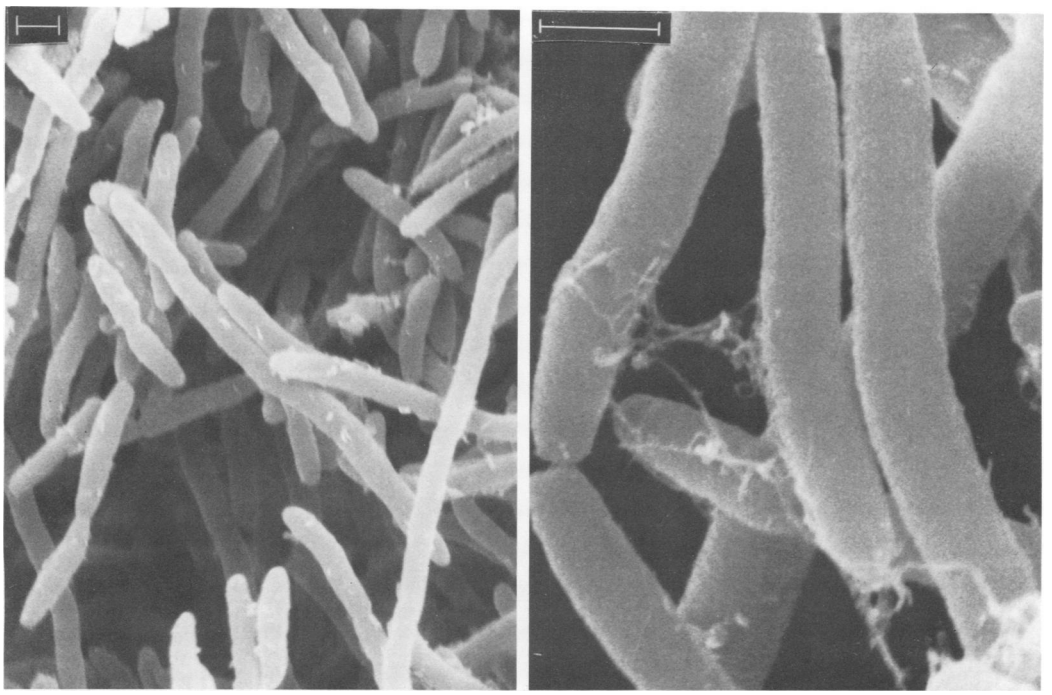

FIG. 3. Scanning electron micrographs of a floc of WR6 cells. Bars = 1 μm.

agitation rates (impeller tip velocity, 1 m/s) appeared to retard selection of ATCC 29191 floc variants in the conventional C30 chemostat. The degree of baffling in the fermentor (i.e., number and position of baffles and probes) and the number and position of the impellers also affected the distribution of flocculent bacteria in the fermentor and hence, selection of this morphological variant in this system.

In summary, the conditions found to be most favorable for floc selection in the chemostat were factors which either promoted (high D, low agitation rate, number and position of impellers, etc.) or exploited (conventional overflow weir) the heterogenous distribution of the mutant bacteria.

Structural analysis of flocculent variants. The morphology of flocculent WR6 cells, as seen by scanning electron microscopy, is shown in Fig. 3. The rod-shaped bacteria were surrounded by an extracellular fibrous material which may function to hold the cells together. This material is similar to the exopolysaccharide matrices which usually surround bacteria growing in natural environments (2, 4, 22). These matrices are thought to be important in adherence of the bacteria to diverse surfaces and other cells. Within the clumps of WR6 cells, there appeared to be sufficient open space around the individual bacteria to allow for free circulation and diffusion of substrates and products in and out of the flocs. High specific rates of productivity have been measured for strain WR6 (8).

To ascertain the chemical nature of the matrix substance, flocs of strains WR6 and 17.3 were exposed to various hydrolytic enzymes. In the presence of a 0.001% (vol/vol) crude cellulase preparation from *Trichoderma reesei* (228 CavU/g; Celluclast 200L Type N; Novo Industries), the clumped bacteria were observed to disperse into single cells within 60 min. Addition of 0.05 to 0.2% carboxymethyl cellulose (Carboxel X-385; Standard Manufacturing Co.) as a potential competitive inhibitor significantly reduced the rate of dispersion. In batch culture, strains WR6 and 17.3 exhibited nonflocculent growth in medium A containing a 0.1% (vol/vol) cellulase preparation and only slight flocculation with a 0.001% preparation.

Taken together, our results suggest that the fibrils surrounding the flocculent *Z. mobilis* cells are cellulosic in composition. Since the crude cellulase preparation used contained low levels of several other hydrolytic enzymes, additional work is clearly required to establish the chemical nature of the fibrils. Several other floc-forming gram-negative bacteria have been reported to synthesize extracellular cellulosic fibrils which appear to mediate flocculation and cell adhesion to surfaces (4).

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