

Clostridium acetobutylicum Protoplast Formation and Regeneration

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Techniques and media for the production and regeneration of stable *Clostridium acetobutylicum* protoplasts are described.

The anaerobe *Clostridium acetobutylicum* has a number of characteristics which suggest that it has much potential in the developing field of biotechnology. The bacterium is already used for the industrial production of acetone and butanol from molasses (10). Further advantages include its ability to utilize pentose sugars (4, 7, 8) and produce a carboxymethyl cellulase and a cellobiase (1). Effluent problems are minimal since after distillation the water is recycled, and the dried stillage can be used as an animal feed supplement (10). The potential of the fermentation system would be greatly enhanced with the development of genetic transfer systems for the bacterium, enabling the use of genetic manipulation techniques for the production of novel products in large fermentors which do not require aeration. As an initial step in the development of genetic transfer systems in *C. acetobutylicum*, we have investigated the formation and regeneration of protoplasts which have been successfully used with other bacteria for the transfer of plasmids by transformation and protoplast fusion (3, 5, 6, 11). Attempts to transfer plasmids to *C. acetobutylicum* by conventional mating and transformation experiments have been unsuccessful to date (unpublished results).

The *C. acetobutylicum* P262 strain which has been described previously (1, 2, 12) was grown in the buffered *Clostridium* basal medium (CBM) of O'Brien and Morris (9). Cultures were incubated at 37°C, and all manipulations were carried out under stringent anaerobic conditions in an anaerobic glove box (Forma-Scientific).

The production of protoplasts by *C. acetobutylicum* P262 was investigated by adding exponential-phase cells to various osmotic stabilizing solutions containing lysozyme (1 mg/ml) (Table 1). In sucrose (0.3 to 0.5 M) and lactose (0.5 M) solutions, 80 to 83% protoplasts were produced after 1 h at 37°C. Other osmotic stabilizers were not as effective in the production of protoplasts by lysozyme. Preconditioning the cells by growth in CBM containing glycine (0.4%, wt/vol) before adding the cells to lysozyme in CBM containing 0.3 M sucrose (CBM+S) increased

the rate and number of protoplast formation, and 90% protoplasts were produced within 15 min. The addition of gelatin (0.5%, wt/vol), palmitic acid (200 nmol), and stearic acid (200 nmol) during the production of protoplasts by lysozyme in CBM+S did not affect protoplast formation. Heavy metals, however, inhibited protoplast formation (unpublished results).

Induction of protoplast formation by sucrose was very rapid, and cells which could not be plated on CBM agar were produced within 5 min in CBM containing 0.5 M sucrose (Fig. 1). Within 5 min >99% of the cells were unable to form colonies on CBM, but did form colonies on the regeneration medium. After 5 min no protoplasts were visible, and they were only observed between 15 and 20 min after adding the cells to CBM containing 0.5 M sucrose.

The number of osmotically sensitive cells and the percentage of regeneration was determined by the difference between the number of colony-

TABLE 1. Production of protoplasts of *C. acetobutylicum*^a

Osmotic stabilizer	Molarity	% Protoplasts
Sucrose	0.5	80
Sucrose	0.3	83
Lactose	0.5	80
MgSO ₄ ·6H ₂ O	0.5	65
MgCl ₂ ·6H ₂ O	0.01	55
Mannitol	0.5	19
Sorbitol	0.5	15
CaCl ₂ ·2H ₂ O	0.01	14
Xylose	0.5	9
CBM		9
Glycerol	0.5	8
Spermidine	0.1	0
NaCl	0.4	0
KCl	1.0	0
Sodium succinate	0.5	0

^a Exponential-phase cells were added to osmotic stabilizing solutions containing lysozyme (1 mg/ml), and the percentage of protoplasts was determined after 1 h by microscopic counts of eight random fields per sample.

TABLE 2. Effect of Ca^{2+} and Mg^{2+} on the stability and regeneration of *C. acetobutylicum* protoplasts

Protoplasting medium	Colony-forming units after dilution in:		% Reversion ^a	% Leakage ^b
	CBM+S	Water		
CBM+S	9.6×10^4	5.5×10^4	43	72
CBM+S + 25 mM Mg^{2+}	3.9×10^5	3.1×10^5	21	36
CBM+S + 25 mM Ca^{2+}	4.0×10^5	3.3×10^5	18	24
CBM+S + 25 mM Mg^{2+} + 25 mM Ca^{2+}	3.1×10^5	4.5×10^4	84	0

^a Reversion was determined by the difference between the number of colony-forming units obtained on the regeneration medium after diluting the protoplast suspensions in either CBM+S or anaerobic water (pH 7.0).

^b Leakage was expressed as the percentage of absorbance at 260 nm in the supernatant fluid; as a reference, the absorbance in a completely lysed protoplast suspension after sonication at maximum amplitude for 5 min was designated as 100%.

forming units obtained on the regeneration medium after diluting the suspensions in either CBM+S or anaerobic water (pH 7.0). The stability of the protoplasts was determined by measuring the leakage into the supernatant fraction of material absorbing at 260 nm. Optimal stability and regeneration (80%) of protoplasts were obtained when the protoplasts were prepared in CBM+S supplemented with MgCl_2 (25 mM) and CaCl_2 (25 mM) (Table 2). The addition of MgCl_2 or CaCl_2 only increased stability of the protoplasts, but decreased their ability to regenerate.

The regeneration of protoplasts was deter-

mined by using many different media; the best regeneration medium which routinely gave 80% regeneration was CBM containing the following (in grams per liter): casein hydrolysate, 10.0; gelatin, 50.0; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 5.1; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 3.7; and agar, 20.0. The percentage of regeneration was increased 5 to 10% either by plating the protoplasts with an equal volume of autoclaved *C. acetobutylicum* cells or by adding bovine serum albumin (0.8%, wt/vol) to the regeneration medium. Techniques used to increase the percentage of regeneration in other bacteria were also investigated, but they did not affect the ability of protoplasts to regenerate. These techniques included plating with sodium dodecyl sulfate-treated, autoclaved cells, plating on membrane filters, pretreatment of protoplasts for 60 min before plating with chloramphenicol, pronase, or trypsin, and preconditioning by growth in CBM+S containing gelatin (12.5%, wt/vol).

Methods for the production and regeneration of viable *C. acetobutylicum* protoplasts have been developed. These techniques will be utilized for the development of host vector systems by using transformation with bacteriophage and plasmid DNA from other *Clostridium* species and other genera. Preliminary studies with bacteriophage DNA indicate that the protoplasts can be used for transformation.

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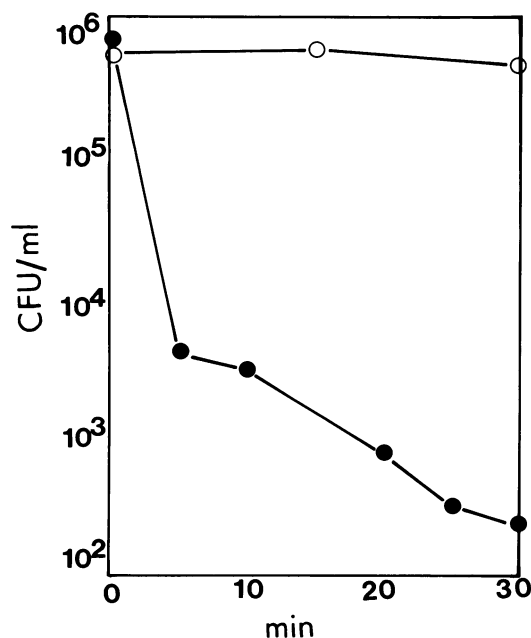


FIG. 1. Rate of production of protoplasts by sucrose and their regeneration by the regeneration medium. Exponential-phase cells were added to CBM containing 0.5 M sucrose and plated on regeneration medium (○) and CBM (●) after different time intervals.

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