FEMS Microbiology Letters 58 (1989) 255–258
Published by Elsevier

FEM 03510

Protoplast formation and regeneration of a thermophilic *Clostridium* sp.

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Received 24 November 1988
Accepted 29 November 1988

Key words: Thermophilic bacterium; Protoplast formation; *Clostridium* sp.

1. SUMMARY

Protoplasts of a thermophilic *Clostridium* sp. were prepared by lysozyme treatment using lactose as osmotic stabilizer. High frequency reversion (3–29.8%) to the bacillary form was obtained on hypertonic rich medium.

2. INTRODUCTION

Thermophilic *Clostridium* species capable of converting cellulose and hemicellulose to chemicals and fuels in a single step are currently of interest in biotechnology. For this reason we searched for cellulolytic thermophiles in different anaerobic environments. An isolate from riverside mud was chosen for taxonomic and genetic studies. We described it as a new species, *Clostridium thermopapyrolyticum* (B. Méndez et al., manuscript in preparation.

A procedure to prepare and regenerate protoplasts is essential for the genetic analysis of Gram-positive bacteria by means of fusion or transformation. Methods for the production and regeneration of protoplasts in *Clostridium* species have already been published for *C. acetobutylicum* [1–3], *C. pasteurianum* [4] and *C. tertium* [5]. However, methods suitable for thermophilic *Clostridium* species are not available, although stable L-phase variants were obtained from *C. thermocellum* [6].

In this work we present a protoplasting and regeneration method for this thermophilic and cellulolytic *Clostridium* sp.

3. MATERIALS AND METHODS

3.1. Strains and culture conditions

C. thermopapyrolyticum UBA 305 was used throughout this study. Cultures were grown in CM4 medium [7] supplemented with glucose 6 g/l (CM4G). Solid medium contained 1% agar (Merck). Stock cultures were kept at 4°C in CM4 medium containing a strip of filter paper (Whatman # 1). The Hungate method [8] was used for liquid cultures. Oxygen-free nitrogen was the headspace gas. Cultures were plated in an anaerobic hood (Coy Laboratory Products) in an 80% N_2, 10% H_2 and 10% CO_2 atmosphere. Incubation temperature was 60°C.

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3.2. Protoplast formation

For protoplast formation, 5 ml of an early exponential phase culture, $OD_{600} = 0.3$, in CM4G were centrifuged and resuspended in one-tenth volume of either SMM buffer [9] or CM4 supplemented with 10% lactose (LPM). Lysozyme was added to a final concentration of 10 μg/ml and the mixture was incubated at 60 °C for 60 minutes.

Protoplastization was followed by phase contrast microscopic counting in 10 random fields.

3.3. Protoplast regeneration

For protoplast regeneration the protoplast suspension was serially diluted in LPM containing 0.1 M (final concentration) *N*-acetylglucosamine and immediately plated on regeneration medium (RM). This hypertonic medium consisted of CM4G supplemented with 1% gelatine, 2% casamino acids, 0.04% bovine serum albumin (BSA), 10^{-2} M $MgCl_2$, 10^{-4} M $CaCl_2$ and 1% agar. The plates were incubated 6–7 days. Lysis-resistant cells were determined plating serial dilutions of the protoplast suspension in distilled water on the regeneration medium. The regeneration frequency was calculated as the ratio of net regenerant colonies (colonies on regeneration medium minus colonies resistant to osmotic shock) per initial cell number.

4. RESULTS AND DISCUSSION

4.1. Protoplast formation

As protoplast viability can be affected by the osmotic stabilizer used [2,10] we evaluated different compounds for their effect on the microorganism's growth before developing a protoplasting medium. Lactose, sucrose and succinate did not present inhibitory effects at concentrations ranging from 0.1–0.6 M. Lactose and sucrose were chosen for further experiments.

Two protoplasting media were tested: SMM and LPM. After 60 min of lytic treatment, similar protoplasting efficiencies were obtained with both solutions. The results of these experiments are shown in Table 1. For routine experiments LPM was chosen.

Table 1

Protoplasting efficiency in different solutions

Protoplasting solution	Original CFU/ml	Lysis resistant CFU/ml	Protoplasting [1] efficiency (%)	Protoplasting [2] efficiency (%)
SMM	2.2×10^6	7.1×10^3	99.9	95
LPM	2.0×10^6	4.9×10^3	99.9	92

[1] The protoplast suspension was serially diluted in distilled water and plated on MR.

$$\%\ \text{protoplasting efficiency} = \frac{\text{original CFU/ml} - \text{lysis resistant CFU/ml}}{\text{original CFU/ml}} \times 100$$

[2] Microscopic observation.

Reilly and Rogers [2] reported that protoplast formation was optimum for *C. acetobutylicum* at a given growth phase. Although most of our experiments were done at early exponential phase, no significant differences in protoplasting or regeneration efficiencies were found for OD_{600} of the starting culture ranging from 0.2–0.6.

4.2. Protoplast regeneration

The regeneration medium was obtained supplementing the growth medium with lactose as osmotic stabilizer, casamino acids to enhance nutrient supply, BSA and gelatine as protective agents and divalent cations to stabilize the protoplasts' membrane.

The results of the regeneration experiments are shown in Table 2.

Table 2

Protoplast regeneration in *C. thermopapyrolyticum* UBA 305

Experiment	Initial CFU/ml [a]	CFU/ml for protoplasts diluted in		% Regeneration [d]
		LPM [b]	Distilled water [c]	
1	2.2×10^6	3.7×10^5	<10	17.1
2	4.1×10^6	1.3×10^5	<10	3.2
3	2.6×10^6	3.3×10^5	1.0×10^2	12.7
4	3.0×10^6	6.1×10^5	1.5×10^3	29.8

[a] CFU/ml of cells before protoplast formation.

[b] and [c] CFU/ml from MR plates.

[d] $\frac{b-c}{a} \times 100$.

We performed some experiments without adding *N*-acetylglucosamine to the dilution medium and the average regeneration frequencies observed were slightly lower than the frequencies obtained with *N*-acetylglucosamine, so this compound was included in the routine procedure.

The omission of any component of the regeneration medium significantly lowered reversion to the bacillary form.

This protocol, developed to form and regenerate protoplasts from *C. thermopapyrolyticum* gives higher regeneration frequencies than those used for other *Clostridium* species. Fusion and transformation experiments using this procedure are presently carried out in our laboratory.

ACKNOWLEDGEMENTS

This work was supported by grants from the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) and from the Fundación Bunge y Born. B.S.M. is a career investigator and S.E.I. had a predoctoral fellowship from CONICET.

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