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PROTOPLAST FORMATION AND REGENERATION FROM *STREPTOMYCES CLAVULIGERUS* NRRL 3585 AND CLAVULANIC ACID PRODUCTION

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ABSTRACT

Protoplasts of the wild type *Streptomyces clavuligerus* NRRL 3585 (ATCC 27064) were formed from spores cultures obtained in the lag, exponential and stationary growth phases by using 0.5% glycine in the culture medium. The protoplasts were obtained by treatment of the cells with lysozyme (EC-3.2.1.17) 40,000 U (1mg/mL), in an osmotic solution for 90 min at 28ºC. The frequency of regenerated protoplasts in the lag phase was $1.7x10^3$ CFU/mL (28.97%), in the beginning of the exponential phase $0.4x10^2$ CFU/mL (31.67%), in the exponential growth phase $2.5x10^3$ CFU/mL (46.30%) and $1.0x10^5$ CFU/mL in stationary phase (48.45%). Antibiotic production and activity of regenerated protoplasts were observed in all phases, except in the lag phase. The protoplast formation and regeneration techniques resulted in a new isolate strain of *Streptomyces clavuligerus* that produced approximately 2.5 fold more clavulanic acid.

Key words: *Streptomyces clavuligerus*, protoplast, clavulanic acid

INTRODUCTION

Streptomyces clavuligerus belongs to a small group of Actinomycetes characterized by beta-lactam antibiotic production and other metabolic intermediates of the biosynthetic pathways (1,2,3,9,10,16,26). Isolated samples of *S. clavuligerus*, which produce at least five antibiotics, including the clavulanic acid has been described by Higgens and Kastner (12). Sermonti and Spada-Sermonti (29) were the first to report about recombination studies on *Streptomyces* using *S. coelicolor*. The protoplast fusion, transformation and improved fermentation features can be used to regenerate strains with increased antibiotic activity (13,15,16,17). The protoplast formation and regeneration are important processes, and they are a major step following genetic manipulations such as fusion and DNA-mediated transformation, which can improve antibiotic production (14,15,21,22,25). The initial procedures of genetic manipulation using *Streptomycetes* were described by Baltz, (4,5) and Okanish *et al.* (28). However, reports of studies on the regeneration of protoplasts from *Streptomyces* are limited (13,16). This paper reports the conditions for protoplast formation and regeneration from *S. clavuligerus* and also relates the improving conditions for clavulanic acid production.

MATERIALS AND METHODS

Microorganisms: *Streptomyces clavuligerus* NRRL 3585 (ATCC 27064), a clavulanic acid producer strain and *Klebsiella pneumoniae* (ATCC 29665) a clavulanic acid sensitive strain were used in this work.

Experimental procedures

Inoculum Preparation: *Streptomyces clavuligerus* was maintained on ISP2 medium modified by the absence of glucose (yeast extract 0.4%; malt extract 1%; agar 2%, pH 7,0), at 28ºC, during 10 days. Spores from a slope were inoculated in 125 ml

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Erlenmeyer flasks containing 25 ml of fermentation medium (FM) (2), modified in the phosphate concentration to 0.435%. Growth was maintained for 24h in orbital shaker at 28ºC and 200 rpm, and was used as inoculum for subsequent experiments.

Clavulanic Acid Production: For the clavulanic acid production was used FM medium and initial inoculum OD_{600} of 0.1, in orbital shaker at 28ºC and 200 rpm, during 96h.

Protoplasts Formation and Regeneration: Cultures described above (preculture) were used to inoculate experimental cultures consisting of 180mL GFM medium (FM medium containing 0.5% glycine) in 1000mL Erlenmeyer flasks to give an initial OD of 0.1 at 600nm. Cultures were incubated in an orbital shaker at 28ºC and 200 rpm for 96h. Samples were collected at intervals of 24h, and centrifuged at 2000x*g* for 10 minutes at 28ºC. The pellets were washed with 20% (w/v) sorbitol solution, and resuspended in 5ml of medium for protoplast formation (MPF) containing lysozyme (EC-3.2.1.17) 1mg/mL (16), modified by the use of sorbitol and TES (n-tris hydroximethyl-2-aminoethan sulphonic) in substitution of sucrose and MOPS (morpholino sulphonic), sterilized by membrane filtration. The samples were incubated at 28ºC for 90 minutes, filtered through glass wool, washed with 5ml of MPF medium and centrifuged at 1000x*g* for 5 minutes at 28ºC. Protoplast formation was accompanied by optical and electron transmission microscopy using the method of Campos-Takaki *et al*. (8). To observe the protoplast regeneration, plates containing 10ml of regeneration medium-RM (2), modified by the absence of MOPS, were inoculated with 0.2 and 0.5mL of the protoplast suspension, and 5mL of regeneration medium-RM were over laid. The Petri dishes were incubated at 28ºC for 5 days. The control of culture was obtained with cells grown in FM medium added of 1.5% agar. The colonies formation was used to estimate the regeneration rate.

Antibiotic Activity: Ten samples with higher antibiotic activity by diffusion disc method (11,23) have been selected for studies of the clavulanic acid production on FM and GFM media. The antibiotic activity was measured by the formation of an inhibition halo during 24, 48, 72 and 96h. The samples were designated in the following format: T_{n-m} where n = culture growth time (1 = 24; 2 = 48; 3 = 72 and 4 = 96h) and m = number of selected samples. Clavulanic acid production was expressed as g of antibiotic per g of biomass, using clavulanic acid as standard.

Analytical Methods: Biomass was determined by measurement of dry weight and related to optical density measurements at 600 nm using a calibration curve. Total proteins were determined by Bradford assay (7), and glycerol by enzymatic method (6).

RESULTS AND DISCUSSION

Effect of growth phase on formation and regeneration of *S. clavuligerus* protoplasts

To determine the optimal conditions for *S. clavuligerus* protoplasts formation, the kinetic of growth and clavulanic acid production in FM and GFM media were investigated. The culture showed a μ_{max} of $0.04h^{-1}$ and generation time of 17.32h. The glycerol consumption was proportional to biomass concentration, and exhibited a V_{max} for consumption of $0.033h^{-1}$. A growth limitation was observed after 60h of fermentation, followed by glycerol exhaustion (Fig. 1A). The growth of *S. clavuligerus* in GFM medium exhibited a μ_{max} of $0.033h^{-1}$ and generation time of 21h. The V_{max} of glycerol consumption was $0.066h^{-1}$. After total glycerol consumption the cells continued to grow for 12h and reached the stationary phase (Fig. 1B). The difference in antibiotic production per gram of glycerol consumed and per gram of biomass, in the two media was not significant (Table 1). However, the yield of antibiotic production per gram of extracellular protein was higher (1.655) in FM medium than in GFM medium (1.185) (Table 1). The influence of protoplasting in *S. clavuligerus* was related to mycelium age. The regeneration of cell wall occurred in crescent percentual and proportional to mycelium age fermentation. The percentage of regeneration was

Table 1. Kinetic of antibiotic activity of *Streptomyces clavuligerus* (wild strain) related to cellular growth in fermentation (FM) and fragilization (GFM) media.

Kinetic Parameters	Fermentation Medium (FM)	Fragilization Medium (GFM)
g Antibiotic / g glycerol consumed	0.026	0.021
g Antibiotic / g biomass	0.052	0.062
g Antibiotic / g extracellular protein	1.655	1.185

Table 2. Total number of protoplats formed from cells of *Streptomyces clavuligerus* and regenerate colonies, and percentage of reversion of protoplast formed during the growth.

Time of Growth (h)	Protoplasts Formed Cells/mL	Not Regenerated Protoplasts Cells/mL	Regenerated Protoplasts CFU/mL	Reversion Percentage (%)
24	$5.8x10^3$	$4.1x10^3$	$1.7x10^3$	28.97
48	$1.2x10^2$	$0.8x10^2$	$0.4x10^2$	31.67
72	$5.4x10^3$	$2.9x10^3$	$2.5x10^3$	46.30
96	$2.1x10^5$	$1.1x10^5$	$1.0x10^5$	48.45

CFU = Colonies Forming Units.

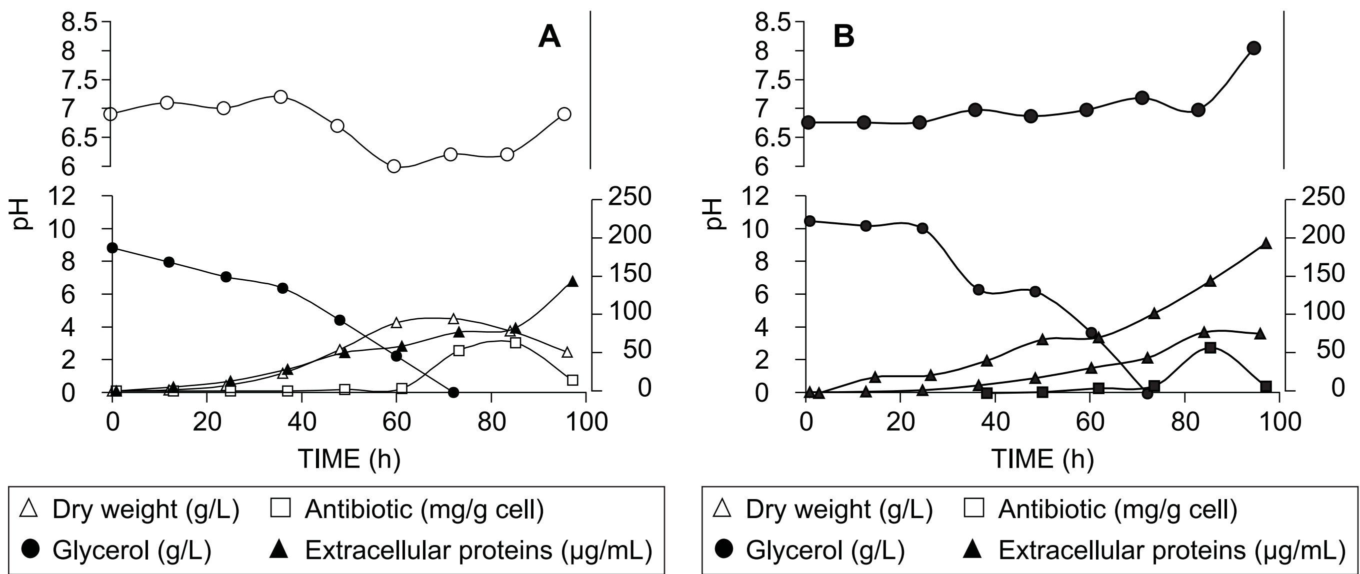

Figure 1. Growth curves, consumption of glycerol, antibiotic production, extracellular proteins and pH curves of *Streptomyces clavuligerus* cultures. A - Culture in FM medium. B - Culture in GFM medium.

higher in the late log phase and in the stationary phase of mycelium growth, where 46.30% and 48.45% reversion percentages were obtained, respectively (Table 2). The protocol used indicates that the age is the most important parameter for formation and regeneration protoplasts frequency. These results are supported by the literature (16,18,20,24). Fig. 2 shows the sequential steps of protoplast formation from *S. clavuligerus*, revealed by transmission electron micrography. The optical microscopy of protoplasts regeneration shows presence of "coccus-like" formations, when compared with the wild sample, and consequent recuperation of the original form of the mycelium (Fig. 3). This phenomenon was also observed by Okanish *et al*. (28).

Effect of protoplasting on antibiotic production by *S. clavuligerus*

Antibiotic activity was studied with forty regenerated colonies of S. *clavuligerus*, using the disc diffusion method (11,23). Ten selected regenerated colonies from each time interval (24, 48, 72 and 96h) were grown in fermentation medium and the antibiotic activity to clavulanic acid production was determined. The highest antibiotic activity/clavulanic acid production was observed in five strains: T_{2-1} and T_{2-8} (48h); T_{3-5} and T_{3-7} (72h) and T_{4-8} (96h). Antibiotic activity/clavulanic acid production was not observed in T_1(24h). The yield of the antibiotic in regenerated samples is shown in Table 3. The antibiotic activity and the clavulanic acid production yield for antibiotic per g biomass obtained for T_{4-8} regenerated sample, showed to be about 2.5 times higher than for the wild strain. These results corroborated those by Ivanova *et al*. (19), who reported the influence of protoplasting and protoplast

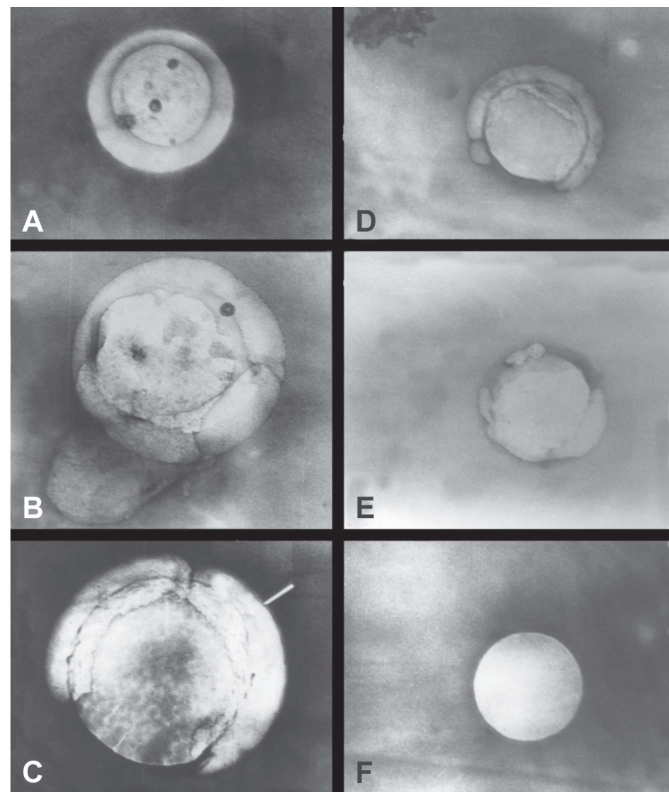

Figure 2. Transmission electron micrographs of protoplasts formation from *Streptomyces clavuligerus.* **A** – Intact cell (17000 X); **B** – Cell in initial process of enzymatic digestion (18000 X); **C**, **D** and **E** – Partially enzyme-digested cell (14400 X, 17000 X and 17000 X, respectively); **F** – Protoplast (17000 X).

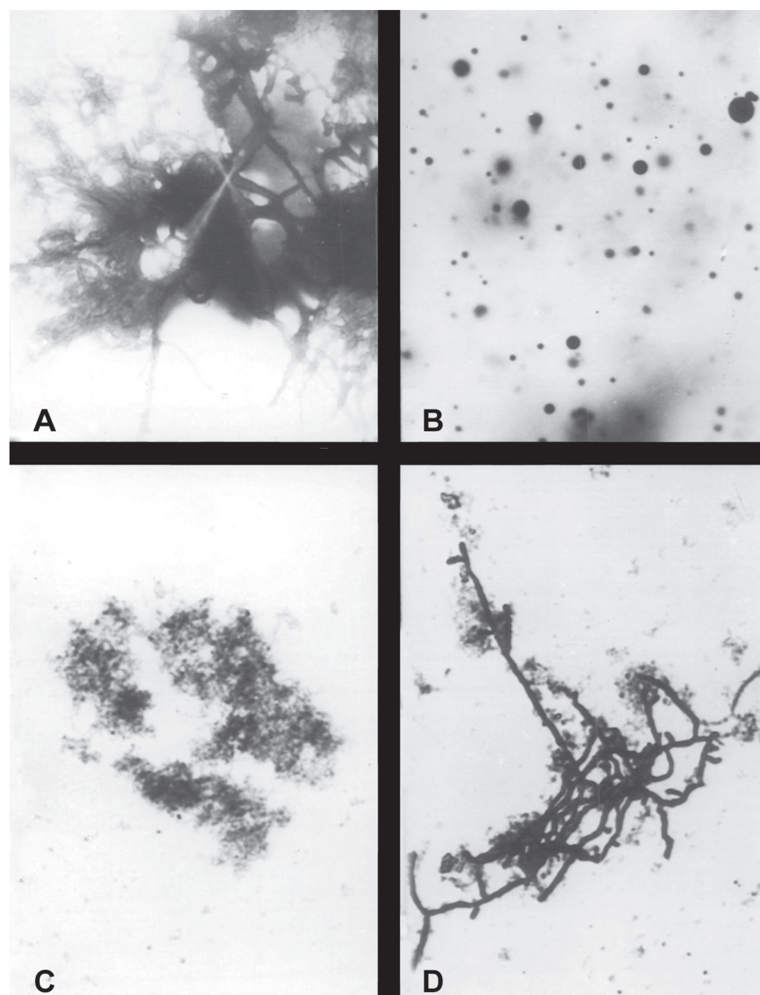

Figure 3. Optical microscopy of protoplasts formation and regeneration from *Streptomyces clavuligerus*. A – Wild strain (FM medium); B – Protoplasts (GMF medium); C and D – Regenerated sample after growth in FM medium for 24h and 48h, respectively (250 X).

Table 3. Clavulanic acid production (g) by biomass (g) of *Streptomyces clavuligerus* wild strain and regenerated cells grown in fermentation (FM) and fragilization (GFM) media.

Samples	Clavulanic Acid Production (g) /Biomass (g)	
	Fermentation Medium (FM)	Fragilization Medium (GFM)
T_{2-1}	0.050	0.042
T_{2-8}	0.031	0.033
T_{3-5}	0.059	0.059
T_{3-7}	NS	NS
T_{4-8}	0.140	0.154
Wild strain	0.052	0.062

NS = not significative.

regeneration on the antibiotic activity. We consider that medium composition and presence of stabilizers influenced protoplast formation and regeneration, but the growth phase is the most important parameter on the process of protoplast regeneration. These results showed an enhanced antibiotic activity, with beneficial effects on clavulanic acid production by the use of an effective protoplasts-regeneration system.

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RESUMO

Formação e regeneração de protoplastos de *Streptomyces clavuligerus* NRRL 3585 e produção de ácido clavulânico

Protoplastos foram formados a partir de esporos da amostra selvagem de *Streptomyces clavuligerus* durante a fase lag, exponencial e estacionária de crescimento, utilizando glicina a 0.5% como meio de cultura. Os protoplastos foram obtidos pelo tratamento das células com lisozima (EC-3.2.1.17) 40.000 U (1mg/mL) em solução osmótica de sorbitol e TES, por 90 min a 28ºC. A freqüência de protoplastos regenerados na fase lag foi de $1,7x10^3$ UFC/mL (28,97%), no início da fase exponencial correspondeu a $0,4x10^2$ UFC/mL (31,67%), no final da fase exponencial observou-se $2,5x10^3$ UFC/mL (46,30%) e para a fase estacionária de crescimento apresentou $1,0x10^5$ UFC/mL (48,45%). A produção do antibiótico e a atividade antibiótica dos protoplastos regenerados foram observadas em todas as fases de crescimento, exceto na fase lag. As técnicas de formação de protoplastos e regeneração resultaram em uma nova linhagem de *Streptomyces clavuligerus* produzindo 2,5 vezes mais ácido clavulânico.

Palavras-chave: *Streptomyces clavuligerus*, protoplasto, ácido clavulânico.

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