FORMATION OF *STREPTOMYCES* PROTOPLASTS DURING CULTIVATION IN LIQUID MEDIA WITH LYTIC ENZYME

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Abstract: Many streptomycetes strains are hardly or not at all transformable via protoplasts, or there is a problem with the regeneration of protoplasts. We found that protoplasts are formed directly in cultivation media under submerged conditions in the presence of lytic enzyme. Actinophage μ1/6 endolysin and lysozyme were used in this study. *Streptomyces* strains were cultivated in several media with glycine and lytic enzyme for 24 and 48h. The highest amounts of protoplasts (about 3 x 10^7 cfu/ml of cultivation medium) together with the highest regeneration (95%) and transformation frequency (about 2 x 10^6 – 10^7 cfu/μg DNA) were obtained reproducibly in YEME medium with high sucrose content. *S. aureofaciens* B96, as hardly transformable strain because of difficulties with protoplast preparation and their further regeneration, was used in this study. The same procedure was applied to *S. lividans* 66 TK24 and *S. coelicolor* A3(2), streptomycetes model strains, to confirm the general use of this method. Moreover, such cultivation process was appropriate for additional quick isolation of either chromosomal as well as plasmid DNA that could be further used in recombinant DNA techniques.

Keywords: *Streptomyces*, protoplasts, transformation, lysozyme, actinophage μ1/6 endolysin

1. Introduction

Streptomyces are unusual among bacteria in growing as mycelial colonies with sporulating aerial hyphae. They are known for degradation of numerous macromolecules and synthesis of a wide range of antibiotics and other commercially important secondary metabolites. The complex life cycle of these bacteria includes formation of substrate mycelia, aerial hyphae and spores (PIGAC and SCHREMPF 1995). The study of the genetics of *Streptomyces* is important not only because of many antibiotics, but also because its differentiation and its regulation of secondary metabolism are of basic interest (OCHI 1982). The preparation and regeneration of protoplasts are major steps following genetic manipulations such as fusion, transfection and transformation of *Streptomyces* species.

Despite there have been reports of "natural" transformation and transfection of *Streptomyces* cultures, there is no such system generally applicable to most species (KIESER *et al.* 2000). Besides transfection and transformation, there have been further means of plasmid transfer described. Intergeneric conjugation, using *Escherichia coli* as a donor, allows constructing and manipulating recombinant plasmids in *E. coli* and subsequently transferring them into *Streptomyces* (PARANTHAMAN and

DHARMALINGAM 2003; HOU *et al.* 2008). Further alternative to using chemicals for promoting an uptake of DNA by cells is electroporation. Protoplasts of *S. venezuelae* and some other streptomycetes, formerly not transformable by the standard protocol, were successfully transformed by electroporation (PIGAC and SCHREMPF 1995). In addition, protoplast fusion is widely used for genome shuffling among interspecies and intergenetic microorganisms (IMADA *et al.* 2002; EL-GENDY *et al.* 2008; XU *et al.* 2008).

Though, the most of aforementioned methods require preparation of protoplasts and their successful regeneration. It has been found that the regeneration frequency of the protoplasts varies according to the species used and high regeneration frequency is limited to a narrow range of *Streptomyces* species (SHIRAHAMA *et al.* 1981, IMADA *et al.* 2002). For the further study of genes in various *Streptomyces* it was necessary to develop procedures for efficient protoplast regeneration and plasmid transformation remembering also the potential presence of the restriction-modification system (MATSUSHIMA and BALTZ 1985; MATSUSHIMA *et al.* 1987; GODÁNY *et al.* 1991; MUCHOVÁ *et al.* 1991).

The aim of this study was to try the effect of lytic enzymes, mainly actinophage μ1/6 endolysin, on protoplast preparation during cultivation under submerged conditions. Up to now, this kind of procedure has not been reported. This work describes an easy and effective method for preparation of protoplasts from *Streptomyces* species, mainly *Streptomyces aureofaciens* B96, by cultivation in liquid medium containing lysozyme or actinophage μ1/6 endolysin. Furthermore, cultivation with lytic enzyme facilitates the isolation of chromosomal and plasmid DNA.

2. Materials and methods

2.1 Bacterial strains and plasmids

Streptomyces strains used in this study: *S. aureofaciens* B96 (Collection of Microorganisms, Institute of Molecular Biology SAS Bratislava); *S. coelicolor* A3(2), and *S. lividans* 66 TK24 (HOPWOOD *et al*. 1985). Shuttle promoter-probe vector pKJ2 (NAZAROV *et al.* 1990) was used as a control for transformation of protoplasts.

2.2 Culture conditions and solutions

Streptomyces sp. were cultivated in TSB (tryptone soya broth powder, Oxoid; containing 0.7% glycine), TSSB (TSB + 10.3% sucrose, 0.7% glycine, Serva), NB (nutrient broth No. 2, Imuna Šarišské Michaľany; containing 0.7% glycine), NBS (NB + 10.3% sucrose and 0.7%glycine), YEME (prepared according to KIESER *et al*. 2000, containing 0.7% glycine), sterile P buffer (prepared according to KIESER *et al*. 2000) and 25% PEG 1000 in T buffer (prepared according to KIESER e*t al*. 2000), lysozyme water solution (Applichem, 40mg/ml, filter sterilized), actinophage μ1/6 endolysin (FARKAŠOVSKÁ *et al*. 2003, 5mg/ml), appropriately dried plates No. 16M (10.3% sucrose, 1.5% dextrin, 0.001% urea, 0.005% NaCl, 0.005% K_2HPO_4,

0.005% $MgSO_4$, 0.5% peptone, 0.1% beef extract, 2ml/l trace element solution (KIESER *et al*. 2000) and 3% agar, pH 7.2).

Solutions used further in this study: thiostrepton (Calbiochem, 50mg/ml in DMSO), TE buffer (10mM Tris-HCl, pH 8, 1mM EDTA), neutral phenol-chloroform (1:1, equilibrated with TE buffer, pH 8), acid phenol-chloroform (1:1, equilibrated with water), 3M sodium acetate pH 4.8.

2.3 Growth of Streptomyces mycelium for protoplasts preparation

10 ml of sterile media containing lysozyme (SERVA, final concentration 1mg/ml and 2mg/ml, respectively) or actinophage μ1/6 endolysin (final concentration 0.15mg/ml and 0.3mg/ml, respectively) in 100ml Erlenmeyer flasks were inoculated with 200μl of dense spore suspension (prepared according to KIESER *et al*. 2000). The lytic enzyme (lysozyme or endolysin) was added either immediately with inoculum or after 8h of incubation. Flasks were incubated at 30°C on rotary shaker for 24 and 48 hours while testing the suitable medium for protoplast preparation.

In the next step, 10 ml of TSB with glycine, but without lytic enzyme, was inoculated with 1ml of dense spore suspension. The flasks were incubated at 30°C on rotary shaker for 20 – 24 hours. After that, $1x10^6$ of colony forming units (cfu) were used as inoculum to 25 ml of YEME medium with lytic enzyme (apart from the control flask) and incubated for 24 and 48 hours.

The culture medium was poured into screw cap bottle and centrifuged. The supernatant was discarded and the pellet was suspended in 10 ml of P buffer. Suspension was filtered through cotton wool (using a filter tube) and transferred into a plastic tube. The protoplasts were collected by centrifugation at 2800 rpm. Pellet was resuspended in 500μl of P buffer.

2.4 Transformation of protoplasts

Transformation of protoplasts with pKJ2 plasmid DNA was done according to HOPWOOD *et al*. (1985) rapid small-scale procedure and plated on 16M agar plates (regeneration medium, 10.3% sucrose, 1.5% dextrin, 0.001% urea, 0.005% NaCl, 0.005% K_2HPO_4, 0.005% $MgSO_4$, 0.5% peptone, 0.1% beef extract, 2ml/l of trace element solution (HOPWOOD *et al.* 1985) and 3% agar, pH 7.2). After 20h incubation at 30°C the agar plates were overlaid with 1ml of thiostrepton (300μg/ml water solution), dried and incubated at 30°C for 2 – 3 days.

2.5 Isolation of Streptomyces "total" DNA

1ml of medium after 24 and 48h incubation was centrifuged and neutral phenol-chloroform was added to supernatant. The extraction from phenol-chloroform was repeated until the white interface was seen. The DNA was precipitated from supernatant by adding 1/10 volume of 3M sodium acetate and 1 volume of isopropanol. After incubation at room temperature and centrifugation, the pellet was

washed by 96% ethanol and dried slightly. Then, the pellet was dissolved in 50µl of TE buffer and the presence of isolated DNA was confirmed by 0.9% agarose gel electrophoresis using ethidium bromide detection.

2.6 Isolation of plasmid DNA

For this part of work, pKJ2 transformants of *S. aureofaciens*, *S. lividans* and *S. coelicolor* were used as inoculum. 1ml of culture medium after 24 and 48h incubation was centrifuged and acid phenol-chloroform was added to supernatant. Mixture was vortex mixed and centrifuged. The supernatant was removed, leaving the white interface behind. 1/10 volume of 3M unbuffered sodium acetate and 2 volumes of 96% ethanol was added to supernatant and mixed. Mixture was incubated at –20°C and then centrifuged. The pellet was dissolved in 500µl of TE buffer and the solution was extracted by neutral phenol-chloroform until no white interface was seen. 1/10 volume of 3M unbuffered sodium acetate and 2 volumes of 96% ethanol was added to supernatant and mixed. Mixture was incubated at –20°C and then span. The pellet was dissolved in 25µl of TE buffer, visualized in 0.9% agarose gel electrophoresis using ethidium bromide detection and 5µl of such solution was used for transformation into protoplasts.

3. Results and discussion

3.1 Protoplasts preparation

Polyethylene glycol (PEG)-mediated plasmid transformation of protoplasts had allowed the rapid development of gene cloning in various *Streptomyces* species, particularly in *S. lividans* 66, *S. ambofaciens*, *S. coelicolor* A3(2), *S. fradiae*, and *S. rimosus*, as well as in some others (PIGAC and SCHREMPF, 1995). This transformation procedure has been generally applicable to several *Streptomyces* species, although it has been necessary to optimize growth and establish the optimal conditions for protoplast formation and regeneration. Moreover, the transformation of the fragile protoplasts has been tedious and frequently not reproducible; thus, numerous *Streptomyces* strains could not be proven to be transformable.

Preparation of protoplasts is essential for further genetic manipulations, such as transformation, transfection (KIESER *et al.* 2000), electroporation (PIGAC and SCHREMPF, 1995) or protoplast fusion (XU *et al.* 2008), known so far. Furthermore, there has been considerable interest in the use of the intergeneric conjugation as a means of plasmid transfer, using *E. coli* as a donor (VOEYKOVA *et al.* 1998; HOU *et al.* 2008).

Despite all possibilities, transformation stayed the most applied method, and many problems with transformation and protoplast regeneration of *Streptomyces* species still preserved. Strain development and genetic analysis of several important streptomycetes has been hindered by the apparent lack of natural fertility, the lack of transduction or transformation, and by restriction-modification systems. Restriction endonuclease activities, having role in the protection of the bacterial genome, can

complicate genetic manipulation of the bacterium by affecting transformation efficiency and stability of recombinant DNA (APICHAISATAIENCHOTE *et al.* 2005). Several methods have been designed in an attempt to improve protoplasts preparation and regeneration in *Streptomyces*, including glycine treatment of mycelia, use of mixture of lytic enzymes, and supplementation of metal ions and osmotic stabilizers (YANG and LEI, 2001). Our studies showed that cells could be transformed at a high frequency when the recipient *Streptomycetes* protoplasts are formed by treatment with lytic enzyme during the cultivation.

In this work, *S. aureofaciens* B96, hardly transformable *Streptomyces* strain, and model strains, *S. coelicolor* and *S. lividans*, were used for setting the conditions for protoplast preparation during the cultivation process. *Streptomyces* under submerged conditions enter the log phase before 24h, stationary phase between 24 and 96 h, and declining phase after 96h of incubation. It was reported that *Streptomyces* protoplast formation was high in the early stationary phase and decreased in the late stationary phase (KIESER *et al.* 2000; YANG and LEI, 2001). In addition, protoplast formation also increased in the decline phase, likely due to the autolysis of mycelia and partial damage of the cell wall. RODICIO *et al.* (1978) found that protoplasts were yielded in young mycelia treated with lysozyme, even when the glycine was not added during the growth period. However, protoplast formation in old mycelia required the presence of glycine during the cultivation. Therefore, mycelium grown at the late log to the middle stationary phase treated with lysozyme was effective for protoplast preparation. In our study, glycine was used in all media as a factor increasing susceptibility to the action of agents degrading the cell wall. *S. aureofaciens* was cultivated in various media with addition of lytic enzyme. The numbers of formed protoplasts, counted in Bürker counting chamber, are shown in Tables 1 and 2.

Table1. Effect of lytic enzymes on number of *S. aureofaciens* B96 protoplasts in various media. Lytic enzymes were added to media after 8h of incubation. Protoplast concentration is in cfu/ml of medium.

Medium	Lysozyme				Endolysin			
	1 mg/ml		2 mg/ml		0.15 mg/ml		0.3 mg/ml	
	24h	48h	24h	48h	24h	48h	24h	48h
NB + gly	--	--	--	--	--	--	--	--
NBS + gly	2×10^6	2×10^6	--	--	4×10^6	4×10^6	4×10^6	4×10^6
TSB + gly	4×10^6	2×10^6	4×10^6	2×10^6	8×10^6	8×10^6	8×10^6	8×10^6
TSSB + gly	12×10^6	8×10^6	14×10^7	12×10^6	16×10^7	14×10^6	16×10^6	14×10^6

There were too few protoplasts formed, when the lytic enzyme was added immediately into the cultivation media (less than100 cfu/ml). More effectual results were when the lytic enzymes were added after 8h incubation of *Streptomyces* culture (Table 1; 2×10^6-1×10^7 cfu/ml). Under these conditions, the best results were acquired in TSSB medium ($12–14\times10^6$ cfu/ml), where additional sucrose was added to form hypertonic environment. Moreover, if NB and NBS media were compared, there were no protoplasts observed in media without any osmotic stabilizer. However, the formation of protoplasts was influenced by the composition of cultivation media. NB

is a complex cultivation medium and was not suitable for *Streptomyces aureofaciens*. Optimal protoplast formation was obtained by treatment of mycelium with lytic enzyme during cultivation in YEME medium (1000mM sucrose, Table 2). However, *S. aureofaciens* grew with much difficulties in such hypertonic medium and needed to be pre-cultivated overnight in TSB medium, where its growth was very extensive.

Table 2. Effect of lytic enzymes on number of *S. aureofaciens* B96 protoplasts in YEME medium. The inoculum was 20-24h fresh culture pre-cultivated in TSB+gly medium. Protoplast concentration is in cfu/ml of medium.

Time of cultivation	Lysozyme		Endolysin	
	1 mg/ml	2 mg/ml	0.15 mg/ml	0.3 mg/ml
24h	24×10^6	26×10^6	29×10^6	30×10^6
48h	26×10^6	29×10^6	31×10^6	31×10^6

The concentrations of lytic enzymes were selected according to preliminary studies in our laboratory and related to lysozyme they were consistent with the results of KIESER *et al.* (2000) standard protocols for protoplast preparation. Mycelium was grown in YEME medium with glycine for 48 hours. For comparison, protoplasts were prepared also by procedure described in KIESER *et al.* (2000) with 1mg/ml of lysozyme for *S. lividans* or *S. coelicolor* and 2mg/ml of lysozyme *S. aureofaciens* (Table 2), respectively. The amounts of prepared protoplasts were comparable to those formed by cultivation directly with lytic enzyme in YEME medium (approximately 50×10^7 cfu/ml in the case of *S. lividans* or *S. coelicolor*, and 30×10^7 cfu/ml in the case of *S. aureofaciens*). The reproducible results from liquid cultivation with lytic enzymes are summarized in Tables 2 and 3. There was not a significant difference among the final protoplast amounts, when the different concentrations of lytic enzyme were used directly in media during the cultivation process. In this study, even the lower concentration (1mg/ml of lysozyme and 0.15 mg/ml of actinophage endolysin) was enough for protoplast formation.

Table 3. Effect of lytic enzymes on number of *S. lividans* 66 TK24 and *S. coelicolor* A3(2) protoplasts in YEME medium. Protoplast concentration is in cfu/ml of medium.

Time of cultivation	lysozyme (2 mg/ml)		endolysin (0.3 mg/ml)	
	S. lividans	*S. coelicolor*	*S. lividans*	*S. coelicolor*
24h	48×10^6	45×10^6	50×10^6	51×10^6
48h	50×10^6	49×10^6	51×10^6	51×10^6

Following the cultivation, protoplasts were centrifuged, washed by P-buffer and filtrated to remove possible mycelia. The important fact was not to dilute cultivation medium because of osmotic instability of protoplasts. After centrifugal concentration at 2800 rpm, protoplasts were ready for transformation. In this state, acquired amounts of *S. aureofaciens* protoplasts were 25×10^9/ml and 25×10^{10}/ml, 5×10^{10}/ml and 4×10^{11}/ml in the case of 1mg/ml and 2mg/ml of lysozyme; 0.25mg/ml and 0.5mg/ml of endolysin used, respectively.

Protoplast regeneration frequency is estimated by the ratio of cell number to protoplast number. It was reported before, that the cell regeneration from protoplasts

of *Streptomyces* depended on the medium components, age of mycelia, dehydratation of plates and culture temperature. It was also found that the presence of non-protoplast mycelium fragments could affect the cellular regeneration from protoplasts (YANG and LEI, 2001). Regeneration medium, No. 16M was suitable for all *Streptomyces* strains tested in this study, and protoplasts were regenerated after 2-3 days of incubation at 30°C. The regeneration frequencies were about 95% for *S. aureofaciens*, 94.3% and 94% for *S. lividans* and *S. coelicolor*, respectively. But in general, because of the diversity of *Streptomyces*, the composition of the regeneration media has to be optimized for each strain.

3.2 Transformation of protoplasts

In past years, polyethylene glycol (PEG)-mediated plasmid transformation of protoplasts had allowed the rapid development of gene cloning in various *Streptomyces* species. Although this transformation procedure is generally applicable to several *Streptomyces* species, it is necessary to optimize growth and establish the optimal conditions for protoplast formation and regeneration. Moreover, the transformation of the fragile protoplasts is tedious and frequently not reproducible; thus, numerous *Streptomyces* strains could not be proven to be transformable (MELLOULI *et al.* 2004).

PEG induces plasmid transformation in *Streptomyces* species apparently by interacting with cytoplasmic membrane structure, thus allowing plasmid DNA uptake (GARCIA-DOMINGUEZ *et al.* 1987). Protoplasts of *S. aureofaciens* B96, in this study, were transformed by pKJ2 plasmid DNA. Thiostrepton resistance marker is well expressed in *S. aureofaciens* and suggested good selection of transformants based on the resistance to this antibiotic. When the protoplasts of *S. aureofaciens* were prepared following the method developed for *S. lividans* and *S. coelicolor* by HOPWOOD *et al.* (1985), the extremely low initial transformation frequency with plasmid DNA was observed (less than 100 transformants per µg DNA). Despite, the frequencies in *S. lividans* and *S. coelicolor* were between 10^6–10^7 cfu/µg of plasmid DNA. Alike in the case of media without or with 300mM sucrose, there was only little transformation frequency with plasmid DNA (5-10%). The highest transformation efficiency was observed when *S. aureofaciens* was cultivated with lytic enzyme in YEME medium (1000mM sucrose, pre-cultivated in TSB medium with glycine). Protoplast numbers after concentration were 25×10^9 cfu/ml and 25×10^{10} cfu/ml in the case of 1mg/ml and 2mg/ml of lysozyme; 5×10^{10}/ml and 4×10^{11}/ml when 0.25mg/ml and 0.5mg/ml of endolysin was used, respectively. Not only the best results in protoplast formation were in such osmotic medium, but also the regeneration of the protoplasts was more than 95% and the transformation frequency was comparable to *S. lividans* and *S. coelicolor* used in this study (in all cases the transformation frequencies were reproducibly about 2×10^6 – 10^7 cfu/µg DNA). These results corresponded to the results published previously for *S. lividans* and *S. coelicolor* (KIESER *et al.* 2000) and the other *Streptomyces* strains (SHIRAHAMA *et al.* 1981; MATSUSHIMA and BALTZ 1985; and more). There was an irrelevant difference in transformation frequency regarding to concentrations of lytic enzymes used in cultivation media and

the type of plasmid. Neither the size nor the origin of the plasmid DNA played the role in this study. The preliminary studies in our lab showed that transformation frequency did not depend on the size of the cloning vector (data not shown). Despite *S. aureofaciens* B96 protoplasts were stable after storing at -70°C under any condition used, they were not transformable any more. They always had to be prepared fresh. Opposite, *S. lividans* and *S. coelicolor* protoplasts, prepared in the same way as aforementioned *S. aureofaciens*, were stable at -70°C in P buffer for at least six months.

3.3 Isolation of "total" and plasmid DNA

There are various procedures of *Streptomyces* DNA isolation (KIESER *et al.* 2000). In general, the first step for DNA isolation is the lysis of the cell walls. They require treatment with SDS or sarkosyl, then incubation with lysozyme at 30° or 37°C with periodical trituration. This step takes usually long time and sometimes not all of the mycelia are lysed. Furthermore, the regeneration and transformation ability of protoplasts depends on the way of preparation in many cases. All DNA isolation methods in this study were simplified procedures according to KIESER *et al.* (2000). They were not such time-consuming, as the step of cell wall lysis was excluded. Visually, except for the YEME with high sucrose content, all media were viscous after cultivation with lytic enzyme. There were only a few protoplasts after cultivation in these media, so we assumed that the most of them broke and intracellular content with DNA split into the medium. After that, no lysis was needed and the whole isolation was based only on extraction from phenol, phenol-chloroform and on alcohol precipitation. If hypertonic medium was used for cultivation, like YEME, it was better to dilute the medium either with water or 10.3% sucrose to let the protoplasts break and split inner content into the medium.

It was also possible to isolate plasmid DNA from such cultivation media. The only difference was the extraction from acid phenol-chloroform at first. Consequently, the plasmid DNAs were purified like total DNA. Plasmid DNAs isolated by this procedure were able to transform repeatedly into *Streptomyces* protoplasts.

According to one's needs, by addition of the lytic enzyme into medium during cultivation it is possible to acquire protoplasts with high regeneration and transformation frequency or isolate chromosomal and plasmid DNA. This procedure is applicable to protoplast preparation from many *Streptomyces* species, even from hardly transformable industrial strains, when high concentration of sucrose, as osmotic stabilizer is added into liquid medium. This method is easier, more effective and not so time-consuming than developing and optimizing new procedures. Additionally, chromosomal DNA and plasmid DNA isolations are very easy and quick, as the cell lysis step is excluded.

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