Standardization of conditions for gene transfer in *S. flavisclerotícus* by transformation and conjugation.

Introduction

Adaptation and/or development of efficient genetic procedures for new species of *Streptomyces* is an exercise that needs to be undertaken because the standard genetic techniques developed for model organism may prove refractory with the new species.

S. flavisclerotícus, the organism of the present study, was selected for genetic characterization for mainly three reasons. One, the strain was not well characterized as a producer of bioactive compounds; second, it produces bioactive compounds against Gram +ve bacteria like *Micrococcus luteus* , *Bacillus subtilis* etc. and third, the genome of *S. flavisclerítícus* potentially codes for aromatic polyketide. The organism belongs to *Actinomycetes* group and superficially resembles fungi; it grows very well in standard *Streptomyces* growth media like tryptone soya broth (TSB) at 30^0C and produces yellow colour compound in media like Soyabean mannitol (SMA), R_2YE, MBA etc. The antibacterial principle could be extracted in organic solvent like ethyl acetate. Since this *Streptomyces* species has not been used previously for genetic work, it was essential to optimize conditions for transformation, conjugation and other genetic manipulations with this organism.

Natural process of gene transfer in *Actinomycetes*, mediated via conjugation is quiet widespread but such sexual exchange is species specific only. Introduction of gene in the cell as free DNA (transformation) or virus mediated (transduction) has been documented in some organisms but the affiance falls with increasing divergence of species involved as homology of DNA sequences decreases.

Transformation in *Streptomyces*: Although "natural" transformation of few *Streptomyces* cultures has been reported (Bibb and Hopwood, 1981), it is not applicable to most *Streptomyces* species. The reason for inefficient transformation and

in turn establishment of foreign DNA is restriction barrier exhibited by most *Streptomyces* species (Cox and Baltz, 1984).

There are several ways in which DNA may be introduced artificially into *Streptomyces*. These include transformation with plasmid, cosmid and chromosomal DNA, transfection with DNA containing a phage replicon, phage transduction of chromosomal and plasmid DNA, interspecies conjugation between two *Streptomyces* species and intergeneric conjugation between *Streptomyces* and *E.coli*.

Until recently, artificial transformation and transfection involved protoplast, but electroporation techniques have now been developed to introduce plasmid DNA into mycelial fragments of some species (Pigac and Schrempf, 1995).

Since calcium chloride mediated plasmid transformation, which is central to transformation in *E. coli*, has not been reported in *Streptomyces*. Other transformation protocols like conjugation, electroporation or PEG mediated transformation have been documented (Hopwood et. al., 1985).

Conjugation: It involves physical cell-cell contact and formation of a filamentous structure connecting the two cells. Transfer of single strand DNA involves the action of nickase at *oriT*, the DNA travels through the pilus and on reaching the recipient cell forms a double stranded plasmid DNA after replication. Since the incoming DNA is single stranded, various restriction enzymes cannot act on it. Those *Streptomyces* species that resist PEG aided transformation, may be transformed by conjugation. Interspecies as well as intergeneric conjugation process has been in use for *Streptomyces*.

- **Interspecies:** Conjugation occurs non- synchronously during several hours of mixed growth. It involves several rounds of recombination. Conjugation occurs only on solid media and never occurs in liquid culture. Genetic mapping is carried out using recombinant progeny from mating. Plasmid sex factor is harbored by many strains and recombination seems to depend on its presence.

- **Intergeneric:** Gram-ve and Gram +ve bacteria can exchange DNA via conjugation, *E. coli* and *Streptomyces* species may exchange DNA between each other. So recombinant plasmid may be constructed in easily manipulatable host *E. coli* and transferred to *Streptomyces*. The vectors developed for conjugation purpose are non- replicative and stably integrate into the chromosome (Bierman et.al.,1992). *OriT* sequences are cloned from RK2 group of plasmid and the transfer function is carried out in trans by *E. coli* donor strain. These conjugation experiments are carried out on the solid substrate as Brownian motion and convection in liquid disrupt the rigid sex pili (Kieser and Hopwood, 1991).

Protoplast transformation in presence of PEG was reported by Bibb et al., 1978 and became a landmark in *Streptomyces* genetics. In light of the fact that *Streptomyces* as a host exhibits strong restriction barrier, establishment of transformed DNA still remains a problem in most *Streptomyces* species.

Table 1: Restriction barrier shown by few *Streptomyces* species

Name of the strain	Antibiotic produced	Problems
S roseofulvus	Frenolicin	Non transformable
S. fradiae	Tylosin	Transformation by general and specific processes not always useful.
S. clavuligenius	Penicillin	Problems in getting transformants from protoplast.
S. coelicolor	Actinorhodin	Highly restricted to methylation at A and C nucleotide.

Effect of Restriction barrier on efficiency of Transformation:

Restriction and modification of DNA have been demonstrated in a wide variety of taxonomically unrelated bacteria. More than 200 restriction endonucleases have been discovered, and in many cases the corresponding methylases have been characterized. In *Escherichia coli*, host-mediated restriction has been shown to prevent the

acquisition of plasmid DNA during conjugation, transformation, and transduction. As DNA transfer during conjugation is in the form of single strand and restriction endonuclease act on double stranded DNA, conjugation is more efficient in microorganism exhibiting restriction system. Transmission of unmodified plasmid by bacterial conjugation is markedly resistant to restriction compared with transfer by transformation. Evasion of type I and II restriction systems involves conjugative transfer of multiple copies of the plasmid (Read et.al, 1992).

In *Streptomyces* species not exhibiting restriction barrier, transformation is still the method of choice as conjugation requires cloning '*oriT*' and it is not always feasible due to unavailability of appropriate restriction sites. Transformation may be carried out by electroporation, or in presence of PEG.

- **Electroporation:** A brief high voltage pulse is applied to protoplast/cell or mycelium and suspension of DNA to be introduced. Application of voltage pulse forms transient membrane pore which favors uptake of DNA. Until recently, *Sterptomyces* transformation by electroporation exclusively involved use of protoplast, but electroporation technique has now been developed to introduce DNA into mycelial fragments (Tyurin et. al., 1995).

- **PEG mediated transformation:** PEG induced plasmid transformation of *Streptomyces* protoplasts has allowed rapid development of gene cloning in several *Streptomyces* species, particularly *S lividans*. Though this method is used for wide range of *Streptomyces* species, the transformation efficiency is low and depends on many factors, one of the most important being restriction ability of the *Streptomyces* species to remove foreign DNA, the other factors being growth phase of the mycelium, media composition, source and molecular weight of PEG, lysozyme concentration and time of treatment, concentration and size of DNA, number of protoplast etc.

  Irrespective of the recipient strain, the basic procedure for introducing isolated DNA is the same; removal of gram positive mycelial cell wall with lysozyme to generate protoplasts, which are exposed to plasmid or phage DNA; in presence of PEG, it enters the protoplast by unknown mechanism. The protoplasts are selected

on antibiotic marker on the plasmid, regenerated into mycelial colonies, which are then scored for transformation. The standard procedure for PEG assisted transformation was documented by Sir David Hopwood in 1995. DNA last propogated in *E. coli* or some other *Streptomyces* is subject to restriction by many hosts including *S coelicolor*, closely related strain; *S. lividans* which is non-restricting is preferred for initial cloning experiments and for expression of many genes in *Streptomyces.*

- PEG mediated transformation procedure is generally applicable to several *Streptomyces* species, but it is necessary to optimize growth and establish the optimal conditions for protoplast formation and regeneration for individual species under a given set of conditions.

  In light of the fact that different *Streptomyces* species exhibit high variability in restriction barrier, growth and protoplastation condition etc., transformation protocol for a particular strain may not be reproducible for the other strain, so it was essential to develop an efficient protocol for *S flavisclerotícus.*

In this chapter different parameters have been examined and standardized which influence the efficiency of plasmid transformation and conjugation in *S. flavisclerotícus.* Conditions affecting PEG assisted transformation protocol and intergeneric conjugation with *E.coli* has been optimized.

Standardization of Transformation with *S. flavisclerotícus*: Conditions and procedure for PEG assisted transformation described for *S.lividans* Hopwood, 1985, was initially followed for *S. flavisclerotícus.* Those conditions gave a good regeneration frequency but the transformation efficiency was low. For efficient transformation, basic protocol of Hopwood et al, 1985, was modified and different conditions were optimized so as to suite the requirements of this strain.

Plasmids pSET152 (integrating vector) (Bierman et. al., 1992) and pIJ699 (episomal plasmid) (Kieser and Melton, 1988) (Fig. 1.1) were used to standardize the protocols of conjugation and transformation in *S. flavisclerotícus* respectively.

Examining for the restriction barrier in *S. flavisclerotícus*:

Several *Streptomyces* spp. are known to possess strong methyl specific restriction barrier, wherein methylated DNA is selectively degraded by the cells barring the unmethylated DNA. To study selectivity in *S flavisclerotícus*, pSET152 was transformed in two strains of *E coli* namely DH5α and ET12567 and amplified plasmid was retrieved. *E coli* ET12567 lacks DNA adenine methylase (*dam*) and DNA cytosine methylase (*dcm*) so the plasmid obtained from this strain was devoid of any methylation. Protoplasts of *S. flavisclerotícus* were transformed with equal amount of pSET152 DNA processed from both the strains. Equal transformation frequency was observed in both the cases, indicative of the unbiased nature of *S. flavisclerotícus* in accepting both, methylated as well as unmethylated DNA with equal efficiency.

Influence of media composition: Following media were tested to study the influence of the growth medium on protoplastation, regeneration and transformation of *S. flavisclerotícus*

1) Tryptone Soya Broth
2) Luria Broth
3) Nitrate Defined Medium
4) YEME with 10% sucrose
5) YEME with 20% sucrose
6) YEME with 34% sucrose

Seed culture was grown in TSB. A scanty and delayed growth was observed when other media were tested for seed culturing. Equal amount (5ml late log phase) of culture was inoculated in 30 ml of each media. Each culture was grown for 24, 36 and 48 hrs at 30^0C.

A dense culture of *S. flavisclerotícus* was observed in TSB, YEME with 10% sucrose and YEME with 20% sucrose.

Profusely grown cultures in these three media was used for protoplastation in P buffer with 1mg/ml lysozyme for 90 min at 30^0C. Complete protoplastation could be obtained only in TSB cultures, whereas incomplete protoplastation could be achieved

in both YEME cultures as evidenced by the presence of unprotoplasted mycelial fragments, owning to dispersed growth in later pair of YEME growth media.

Alteration of the conditions for cultures grown in YEME with 10% and 20% sucrose complete protoplastation could be achieved. The concentration (2mg/ml) and duration of incubation (130 min) of lysozyme treatment. When treated with higher concentration of lysozyme and longer time duration protoplastation was complete and mycelial fragments were not observed.

When equal number of protoplast (10^5) were plated for regeneration from cultures grown in each of three medium it was found that, TSB grown culture treated for 90 min. in P buffer with 1mg/ml lysozyme, regenerated in more number than the YEME grown culture. Protoplastation and regeneration was best in TSB followed by YEME with 10% sucrose > YEME with 20% sucrose. Subsequently for testing the effect of other parameters, TSB grown culture was used.

Table 1: Influence of media composition on growth of *S. flavisclerioticus* culture.

Medium	**Period of growth (36 hrs)**	**Period of growth (48 hrs)**
34% sucrose YEME	-	-
10% sucrose YEME	++	+++
20% sucrose YEME	++	++
TSB	+++	+++
NDM	-	+
LB	-	++

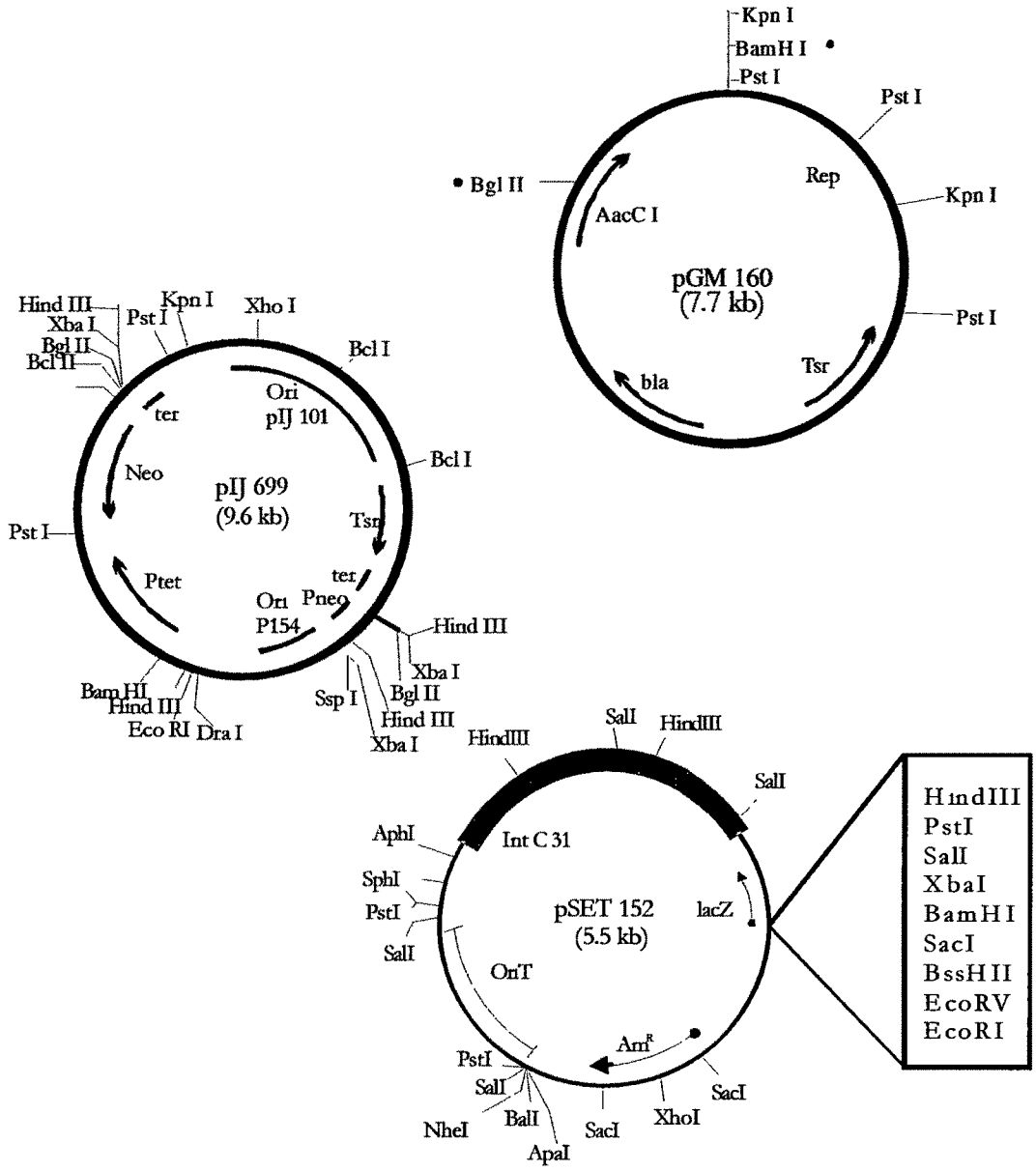

Fig. 1.1: Plasmids used for transformation and conjugation in *S. flavisclerotícus*.

Table 2: Influence of media composition on protoplastation and regeneration

Medium	Protoplast yield (per ml)	Protoplast plated (A)	Protoplasts regenerated (B)	Regeneration efficiency [B/A] X 100.
YEME (10% sucrose)	2.2×10^8	1.1×10^8	3.6×10^6	3.2×10^{-2}
YEME (20% sucrose)	2.5×10^7	1.2×10^7	1.2×10^4	1×10^{-3}
TSB	2.7×10^8	1.3×10^8	6.7×10^5	5.1×10^{-3}

Table 3: Influence of media composition on transformation of protoplast.

Medium	Protoplasts used	Transformants per µg DNA
YEME (10% sucrose)	1.1×10^8	96
YEME (20% sucrose	1.3×10^8	22
TSB	1.4×10^8	130

Influence of culture age: Since TSB was the best medium for protoplastation and regeneration, cultures were grown in TSB for different time period and subjected to protoplastation, regeneration and transformation. Equal number of protoplast and equal concentration of plasmid DNA was used for each experiment. Data in Table 4 shows that older mycelium (72 hrs) released more no. of protoplast than younger mycelium. However, protoplast derived from younger mycelium (36 hrs) regenerated with higher efficiency. When culture was grown up to 84 hrs, a decline in protoplastation as well as regeneration was observed. *S. flavisclerotícus* protoplasts

derived from mycelium harvested at the beginning of the exponential growth phase (36 hrs) gave maximum regeneration frequency and transformation as compared to those obtained from older cultures.

Table 4: Influence of culture age on protoplastation and transformation.

Culture age (hrs)	Protoplasts released (per ml)	Protoplasts plated (A)	Protoplasts regenerated (B)	Regeneration frequency [B/A]X100	Transformants per µg DNA
36	$3.6x10^{8}$	$1.2x10^{8}$	$2.6x10^{5}$	$2.1x10^{-3}$	152
48	$4.2x10^{9}$	$1.6x10^{8}$	$3.3x10^{4}$	$2.0x10^{-4}$	43
60	$3.2x10^{9}$	$1.0x10^{8}$	$6.1x10^{2}$	$6.1x10^{-6}$	0
72	$7.2x10^{6}$	$2.6x10^{6}$	$5.3x10^{1}$	$2.0x10^{-5}$	0

Influence of Temperature: Effect of temperature on protoplastation was monitored at, $4^{0}C$, $30^{0}C$ and $37^{0}C$. Protoplasts produced (released) at $4^{0}C$ regenerated at higher frequency than those produced at $30^{0}C$ and $37^{0}C$. However, number of protoplast and transformants obtained at $30^{0}C$ was higher (Table 5). At $37^{0}C$, both regeneration and transformation was poor. Since at $30^{0}C$ maximum transformants were obtained, it was the temperature of choice for lysozyme treatment and in turn protoplastation.

Table 5: Influence of temperature on protoplastation and transformation.

Temperature	Protoplasts released / ml	Protoplasts plated	Protoplasts regenerated	Regeneration frequency [A/B]x100	Transformants per µg DNA
$4^{0}C$	$7x10^{9}$	$2.6x10^{8}$	$6.2x10^{5}$	$5.1x10^{1}$	33
$30^{0}C$	$3.6x10^{8}$	$7.5x10^{8}$	$3.4x10^{5}$	$4.5x10^{-1}$	162
$37^{0}C$	$2.5x10^{6}$	$4.2x10^{6}$	$5.2x10^{2}$	$1.2x10^{-2}$	12

Influence of lysozyme concentration on protoplastation: Mycelium grown in TSB was treated with three different concentrations of lysozyme, 1-, 1.5- and 2 mg/ ml of P buffer. Time of treatment was 90 min. and yield of protoplast at 2mg/ ml was maximum followed by 1.5mg/ ml > 1mg/ ml, but regeneration frequency was highest at 1mg/ ml of lysozyme (Table 6).

Table 6: Influence of lysozyme concentration on protoplastation and transformation.

Lysozyme concentration mg/ ml	Protoplasts released / ml	Protoplasts plated	Protoplasts regenerated	Regeneration frequency [A/B]x100	Transformants per µg DNA
1	$2.4x10^7$	$1.2x10^7$	$6.2x10^5$	$5.1x10^0$	33
1.5	$6.5x10^8$	$3.2x10^8$	$2.4x10^6$	$7.6x10^0$	137
2	$8.2x10^9$	$4.1x10^9$	$2.2x10^5$	$5.3x10^{-3}$	16

Influence of PEG source, molecular weight and concentration: The source, M_r, and concentration of PEG used to induce DNA uptake was varied in order to optimize PEG usage for transformation. According to earlier reports both 25% and 50% concentration of PEG has been used for transformation. Four different samples of PEG were used at both the concentration. The four PEG sources varied with respect to date of procurement, thus age and batch number.

1) PEG 1000 (sigma, sample1)
2) PEG 1000 (sigma, sample2)
3) PEG 8000 (sigma, sample1)
4) PEG 8000 (sigma, sample2)

Data (Table 7) shows that PEG 1000 sample 1 and 2 both gave best results at 25% concentration wt./ vol. with T buffer.

Table 7: Influence of PEG source and concentration on transformation.

PEG M.W	Source/ Manfacturer	Transformants per µg DNA (25% PEG)	Transformants per µg DNA (50% PEG)
1000 sm1	Sigma	25	4
1000 sm2	Sigma	17	7
8000 sm1	Sigma	14	0
8000 sm2	Sigma	10	0

Influence of protoplast number: Effect of protoplast density on transformation frequency was determined by varying the number of protoplast from 10^4 to 10^{10} Number of transformants obtained was directly proportional to the number of protoplast used. Transformation and regeneration frequency both were directly proportional to protoplast numbers (Table 8).

Table 8: Influence of protoplast number on transformation.

Protoplast plated (A)	Transformants obtained (B)	Transformants per µg DNA	Transformation frequency [B/A]x100
$3x10^5$	18	18	$6x10^{-3}$
$3x10^6$	35	35	$1.1x10^{-3}$
$3x10^7$	70	70	$2.3x10^{-4}$
$3x10^8$	117	117	$3.9x10^{-5}$

Influence of DNA concentration: The effect of plasmid DNA concentration on transformation was examined with pIJ699. Varying amount of plasmid DNA was used for each set of experiment, keeping the protoplast number in the range of 10^9. Frequency of transformation increased from 50 ng to 1µg of plasmid DNA used. Concentration of 1µg of DNA was saturating (Table 9).

Table 9: Influence of DNA concentration on transformation.

DNA concentration (μg)	Protoplasts plated	Transformants obtained
0.05	$3x10^5$	16
0.5	$3x10^5$	72
1	$3x10^5$	137
2	$3x10^5$	130
3	$3x10^5$	119
4	$3x10^5$	123
5	$3x10^5$	127

Standardization of intergeneric conjugation between : Intergeneric conjugation between *E coli* and *Streptomyces* have been found useful for various *Streptomyces spp* Plate conjugation between S17.1 harbouring pSET152, a mobilizable plasmid having *ori*T and *int* DNA, was carried out. Exconjugant frequency of the order of 10^{-3} /cell was obtained which is 10^4 times less than that of *S lvidans,* one of the strains where conjugation is very efficient. In light of these result, it is better to use transformation as gene transfer mechanism for *S. flavisclerotıcus.*

Table 10: Intergeneric conjugation between *E. coli* (S17.1) and *S. flavisclerotıcus.*

Number of spores (A)	Exconjugants (B)	Exconjugants frequency, % of viable recipient [B/A]x100
$5.5x10^7$	712	$1.29x10^{-3}$
$5.5x10^7$	260	$0.47x10^{-3}$
$5.5x10^7$	265	$0.48x10^{-3}$

Discussion

The experiments described in this chapter were used to determine the optimal conditions for plasmid transformation and conjugation of *S flavisclerotıcus*. Similar systems have been used for other *Streptomyces* as well. Optimization of several parameters allowed the best possible transformation frequency under our lab conditions.

Pre-dried (for 2 hrs) R_2YE plates were as regeneration and transformation medium. Sucrose in the medium acts as osmotic stabilizer. To check if the restriction system works and has any effect on transformation, DNA propogated in both DH5α and ET12567 were used for transformation. In both, transformants were obtained in equal number, proving that this species lacks restriction system. Transformation frequency in *S. flavıscleroticus* is better than the conjugation frequency.

Media composition has effect on both seed culture and culture used for protoplastation, regeneration and transformation. Though culture grew profusely in media like LB and NDM, protoplastation was poor in both the cases. Even though cultures grown in YEME with 10% and 20% sucrose has fine and healthy growth it requires more concentration and time of treatment than TSB grown cultures. Also the regeneration and transformation frequency was less as compared to TSB culture. In light of the above observation TSB proved to be the best choice for further work with *S. flavisclerotıcus*.

Growth phase of mycelium was important and showed a major effect on protoplast transformability. Protoplasts generated from 36 hr grown culture were found most efficient for regeneration and transformation. Young cultures yielded less protoplast whereas older cultures yielded less viable protoplasts. Transition period between exponential and stationary phase (36 hrs) yielded efficient regeneration and transformation. As the number of viable protoplast was less in case of older cultures, for the same number of protoplasts, titration or futile binding of DNA to nonviable protoplast affected plasmid uptake and resulted in lesser transformation frequency.

Optimum temperature for transformation was 30^0C, though regeneration was faster for mycelium treated at 4^0C. This may be because spheroplasts, where the cell wall is

only partially digested are generated at 4^0C, which regenerate faster than true protoplast generated at 30^0C. Protoplast can take up DNA whereas spheroplast may not take-up DNA efficiently, so in spite of regeneration being high at 4^0C, transformation frequency is low. At 37^0C both regeneration and transformation was poor. It may be concluded that true protoplast are efficient both in taking up the DNA and regeneration, though spheroplasts regenerates faster, it cannot take up DNA, whereas protoplast from which membrane is removed completely neither takes up DNA nor can regenerate. This explains even the effect of lysozyme used. Higher concentration generates damaged protoplasts whereas lower concentration generates spheroplasts; optimum concentration is required to yield protoplast which can regenerate. Temperature could also affect efficiency of DNA uptake by protoplast, probably lowest temperature inhibiting the process. Temperature change affects the fluidity of the cell membrane, which may influence the DNA uptake.

PEG is known to possess an attribute of acting as volume excluder, the exact mechanism still remains unknown. Different authors have reported different concentration of PEG for efficient transformation of *Streptomyces*. In the present study 25% PEG 1000 from Sigma was found to be best.

Transformation frequency was higher with increasing number of protoplast. Protoplast density in the range of 10^9 was best.

Increasing plasmid DNA concentration showed increase from 50 ng to 1 μg, higher concentration than 1 μg showed slight variation in number of transformants.

Conclusions

The data presented in this chapter shows that though DNA may be transformed in *S. flavisclerotícus* with relatively low frequency as compared to some of the strains of *Streptomyces*, for example, *S. lividans*. Given the efficiency of gene transfer in this strain, the strain is nonetheless amenable to genetic manipulation. The treatments given were to maximize on number of protoplasts, their viability measured in terms of regeneration expected to affect the gene transfer by transformation. Conditions for protoplast formation in the range of 10^9 and regeneration frequency of 4% have been achieved. The effect of growth media and the variables like temperature,

concentration of lysozyme, PEG concentrations were tested not only to maximize for yield of protoplast but also for their being maximally viable, so that maximum number of protoplast take up DNA. Transformation frequency in the range of 10^3/ μg of DNA has been routinely obtained. We did encounter condition where yield of protoplast was less but their viability was good, for eg. in TSB grown culture. On the contrary, YEME + 20% sucrose grown culture produced maximum number of protoplasts however, the viability was discouragingly poor.

Results obtained in the standardization of gene transfer conditions clearly indicate that the transformation/ conjugation method for introduction of DNA may not be as efficient as that for model organisms, *S coelicolor* and/or *S. lividans*. Given the availability of *E coli – Streptomyces* shuttle vectors for efficient manipulation of cloned DNA in *E. coli*, the relatively low efficiency of gene transfer in *S. flavisclerotícus* is hardly a handicap for undertaking studies on genetic characterization of antibiotic biosynthetic genes in *S. flavisclerotícus*.

S flavisclerotícus was found to maintain the plasmid pIJ699 stably but not pGM160 (Muth et.al.,1989) probably due to poor function of the *Streptomyces* replicon DNA. Transformation frequency was influenced by age of culture, temperature of protoplastation, number of protoplasts and DNA, source and concentration of PEG. The relative ease of protoplastation, regeneration, transformation, lack of apparent restriction barrier, stability and maintenance of some plasmids, indicate that *S. flavisclerotícus* is conducive to molecular cloning.