http://www.pjbs.org

PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 10 (24): 4527-4530, 2007
ISSN 1028-8880
© 2007 Asian Network for Scientific Information

Mutation of *Streptomyces griseoflavus* in Order to Obtain High Yield Desferrioxamine Producing Fused Cells

A. Akbarzadeh, D. Norouzian, A. Farhangi, M.R. Mehrabi, M. Shafiei,
D. Zare, Z. Saffari, M. Mortazavi, M. Mardaneh and Z. Nemati
Department of Pilot Biotechnology, Pasteur Institute of Iran, Tehran, 13169-43551, Iran

Abstract: *Streptomyces griseoflavus* PTCC 1130 was mutated by UV irradiation. Two mutants were obtained (C7031 and S7011). These two mutants were able to produce desferioxamine. Desferrioxamine was extracted from the culture broth of the two mutated strains and the thin layer chromatogram of the products showed the R_F values of 0.461, 0.463 and 0.456 for S7011, C7031 and the standard, respectively. The protoplasts of mutated *Streptomyces griseoflavus* were isolated and fused together. Total numbers of 58 fusions were obtained and only 17 fusions showed significant resistance to sodium azide and crystal violet. In terms of production of desferrioxamine only fusion PF9 and PF10 increased 68.3 and 81.8% desferrioxamine production as compared to parent strain (PTCC 1130), respectively.

Key words: *Streptomyces griseoflavus*, desferrioxamine, protoplast fusion, mutation

INTRODUCTION

Microorganisms can be called as cell factories since they perform numerous and different biochemical reactions in their metabolic pathways leading to the synthesis of valuable compounds in response to their nutritional requirements. These compounds are of medicinal, industrial and commercial values and importance. One of the most important chemicals which are of therapeutic value is desferrioxamine (Silvia *et al*., 1992; Alia *et al*., 1998). This is an iron chelating agent and is employed in the treatment of iron overload and pathological iron deposition in humans (Paul, 2006; Petter, 1988). Siderofore desferrioxamine is synthesized as secondary metabolite by *Streptomyces* species in response to the bioavailabity of iron which is very important nutrient for the growth of microorganisms (Kazuki *et al*., 2005). Despite the abundance of iron in the soil but its availability is low as it is present in the form of insoluble complexes (Siebner *et al*., 2004). This has made the organisms to develop iron-scavenging system based on the synthesis of siderophores (Francisco *et al*., 2003). Different species of *Streptomyces* can naturally produce this valuable compound which can also be used in the treatment of malaria (Victor *et al*., 1992). Hence, this compound is produced naturally by *Streptomyces* species in low quantity that is not economically feasible to manufacture it at large scale. To circumvent such, research is in progress to obtain the genetically engineered strain/s which is a high yield desferrioxamine producer and makes easy the down stream processing too. In this paper attempts are made to mutate S*treptomyces griseoflavus* PTCC 1130 and then fuse the protoplast of the mutated S*treptomyces griseoflavus* PTCC 1130 in order to obtain a high yield desferrioxamine producing fusion.

MATERIALS AND METHODS

Organism: *Streptomyces griseoflavus* PTCC 1130 was obtained in lyophilized form from Persian Type Culture Collection, IROST-Tehran, Iran.

Desferrioxamine production in solid and liquid medium: After reviving the organism, spores of *Streptomyces griseoflavus* PTCC 1130 were plated on Des4 medium containing per L, dextrin 20 g, Na_2HPO_4 0.5 g, $Mg(SO_4)_2 \cdot 7H_2O$ 0.25 g, $Zn(SO_4)_2 \cdot 7H_2O$ 0.05 g, l-methionine 0.1 g, l-lysine 0.25 g, d-manitol 20 g, $CaCO_3$ 5 g, aspargine 12 g and l-threonine 0.1 g and agar 35 g. The plates were incubated at 29±1°C for 4 days. After the incubation period, Whatmann paper No. 1 was dipped in a solution of 0.5% (w/v) ferro-ammonium sulfate prepared in 1% H_2SO_4. This was then overlaid onto the plate, the presence of brown zone around and depth of the colonies indicated the ability of organism to produce desferrioxamine.

Corresponding Author: A. Akbarzadeh, Department of Pilot Biotechnology, Pasteur Institute of Iran, Tehran, 13169-43551, Iran
Tel: +98 21 6696 8856/6646 5406 Fax: + 98 21 6646 5132

Production medium: *Streptomyces griseoflavus* was grown in a medium containing per L, soy bean flour 20 g, d-mamitol 20 g double glass distilled water 1000 mL, pH was adjusted to 7.2-7.6 by KOH after autoclaving. The amount of desferrioxamine was estimated calorimetrically at 430 nm by ferro-ammonium sulfate reagent using un-inoculated production medium as blank. The standard curve was plotted by using standard desferrioxamine at the concentration ranged from 0.01 to 0.1 mg mL^{-1}.

Mutation of *Streptomyces griseoflavus* PTCC 1130: *Streptomyces griseoflavus* was mutated by UV irradiation and the mutated *Streptomyces griseoflavus* was identified by its resistance to sodium azide and crystal violet. Plates containing MYA medium each were spread with 100 μL of *Streptomyces griseoflavus* spore suspension (10^6 spores mL^{-1}). The plates were irradiated (at dark) with UV light at the distance of 15 cm and the power of 15 W. The unreacted and reacted plates were incubated at 29±1°C for 7 days.

Isolation of protoplast: Spore suspension of mutated *Streptomyces griseoflavus* (0.2 mL) was diluted with 40 mL of MYB medium. This was then sonicated for 20 sec and divided into 2 parts. One part was suspended in a sterile solution containing per 700 mL of distilled water; sucrose 103 g, K_2SO_4 0.25 g, $MgCl_2 \cdot 7H_2O$ 2.03 g. This was then centrifuged at 2500 rpm for 30 min. Finally, the precipitate was dissolved in 1 mL of the above solution containing 1 mg mL^{-1} of lysozyme. This was allowed to react at room temperature on a shaker slowly.

Protoplast fusion: Protoplast suspension (0.1 mL) was centrifuged at 2500 rpm for 30 min. The precipitate was suspended in 200 μL of the above solution which contained 50% (w/v) PEG 1000. The suspension was kept in room temperature for 1 min, then 10 mL of the above solution without PEG 1000 was added and that was centrifuged as mentioned. The precipitate was suspended in an appropriate solution and the numbers of the protoplasts were counted on Neobar slide. The protoplasts were cultivated on soft medium agar containing either sodium azide or crystal violet and both. Blank medium did not contain inhibitors.

RESULTS AND DISCUSSION

Desferrioxamine was produced by *Streptomyces griseoflavus* PTCC 1130 in solid and liquid media. The production of siderophores desferrioxamine in the former medium was qualitatively shown by immersing Whatmann paper No. 1 in ferrous-ammonium sulfate solution and then overlaying the paper over the colonies. The presence of brown zone around and depth of the bacterial colonies indicated the ability of *Streptomyces griseoflavus* to synthesis desferrioxamine. To quantify desferrioxamine production, *Streptomyces griseoflavus* PTCC 1130 was grown in a liquid medium, the trend in the synthesis of desferrioxamine was followed by measuring the amount of desferrioxamine calorimetrically and the dry weight of *Streptomyces griseoflavus* PTCC 1130 was also obtained. Irradiation by ultra violet is known to be a good tool in mutating the organisms but the optimum time of irradiation is of prime importance that allows 1-5% of the cells to remain alive. Henceforth, 7 sec ultra violet irradiation was observed to be optimum to get 3.06% live *Streptomyces griseoflavus* PTCC 1130 cells. Sodium azide and crystal violet inhibit the growth of *Streptomyces griseoflavus*, Table 1 and 2 show the mean value of the spores remained in media containing (separately) sodium azide and crystal violet at the concentrations ranged from 0.0005 to 0.01% and 0.0001 to 0.001% (w/v), respectively. By the data obtained (Table 1, 2), it was decided to obtain mutants which are resistance to sodium azide and crystal violet at 0.003% (w/v) concentration. Therefore, 47 and 11 colonies were obtained in media containing

Table 1: Comparison of the growth of *S. griseoflavus* on MYA medium containing different concentration of sodium azide

Concentration of sodium azide (w/v)	Mean of Ist attempt (%)	Mean of 2nd attempt (%)	Mean of 3rd attempt (%)
0	3.164	7.158	161.7
0.005	3.730	3.690	71.7
0.001	7.300	3.330	31.7
0.002	0.00	0.00	0.0
0.003	0.00	0.00	0.0
0.004	0.00	0.00	0.0
0.005	0.00	0.00	0.0
0.006	0.00	0.00	0.0
0.007	0.00	0.00	0.0
0.008	0.00	0.00	0.0
0.009	0.00	0.00	0.0
0.01	0.00	0.00	0.0

Table 2: Comparison of the growth of *S. griseoflavus* on MYA medium containing different concentration of crystal violet

Concentration of crystal violet (w/v)	Mean of Ist attempt (%)	Mean of 2nd attempt (%)	Mean of 3rd attempt (%)
0	161.0	157.7	159.3
0.005	ND	ND	ND
0.0001	45.7	49.3	46.7
0.0002	0.0	0.0	0.0
0.0003	0.0	0.0	0.0
0.0004	0.0	0.0	0.0
0.0005	0.0	0.0	0.0
0.0006	0.0	0.0	0.0
0.0007	0.0	0.0	0.0
0.0008	0.0	0.0	0.0
0.0009	0.0	0.0	0.0
0.001	0.0	0.0	0.0

0.003% sodium azide and crystal violet (separately) when the spores were irradiated by UV for 7 sec. The lived colonies were designated as S7011-S7047, C7010-C7011, respectively. By transferring the above medium onto MYA medium which did not contain any inhibitors, nine colonies from sodium azide group (S7013, S7016, S7014, S7019, S7021, S7029, S7033, S7034 and S7035) remained alive and only two colonies of the other group (C7012 and C7017) showed very poor growth on MYA medium. Selected mutated and inhibitor resistance strains of *Streptomyces griseoflavus* were plated onto solid Des4 medium in order to screen out their ability to produce desferrioxamine. The selection was based on the intensity of brown color and the diameter of the zones (Fig. 1), in this way mutated strains of *Streptomyces griseoflavus* S7011 and C7031 were selected according to the above criteria and their ability to produce desferrioxamine in liquid medium was studied. Thin layer chromatogram pattern of extracted desferrioxamine from the liquid culture of *Streptomyces griseoflavus* S7011 and C7031 showed the R_f values of 0.461, 0.463 and 0.456 for S7011, C7031 standard, respectively (Fig. 2). These two high yield desferrioxamine producing strains were employed to obtain a recombinant *Streptomyces griseoflavus* by protoplast transfusion technique. Strains S7011 and C7031 were cultured in MYB containing 0.5% (w/v) glycine for 40 and 60 h, respectively. Then the mycelia of S7011 and C7031 were sonicated with the power of 70 watts for 50 and 70 sec, respectively and treated with 1 mg mL^{-1} lysozyme for 40 and 25 min, respectively to yield enough entire cell wall-free protoplasts. The protoplasts of *Streptomyces griseoflavus* C7031 and S7011 were obtained according to published procedure of (Matsushima and Baltz, 1986). Only a portion of cells can regenerate and form colonies, while some of these colonies are resulted from cells that, more or less, carry partially/completely their cell wall component. To identify the percentage of true protoplast, which could be regenerated and the percentage of regeneration, certain number of each strains protoplasts were cultured on hypertonic (regenerative) and non-hypertonic (regular MYA) media. Then the number of formed colonies on both types of plates were counted and divided by the number of plated protoplasts. Ultimately it was distinguished that in S7011, 47.5% of cells could be regenerated while 31.7% of them were true protoplasts. It meant that 66.7% of cells yielded true protoplasts and could be regenerated successfully. Those percentages for strain C7031 were 39.4, 28 and 71.7%, respectively. According to them, the average output of protoplast formation was (66.7+71.7%)/2 = 68.9%. As soon as protoplasts were prepared, they had to be fused. The most common reagent for fusion of *Streptomycetes* protoplasts is polyethylene glycol (PEG), whose various molecular weight have been used in different concentrations, among them, PEG 1000 in concentration of 50% (w/v) is the most recommended and efficient one (Hopwood and Chater, 1984; Matsushima and Baltz, 1986).

Fig. 1: Zones showing production of desferrioxamine by *S. griseoflavus* C7031 and S7011

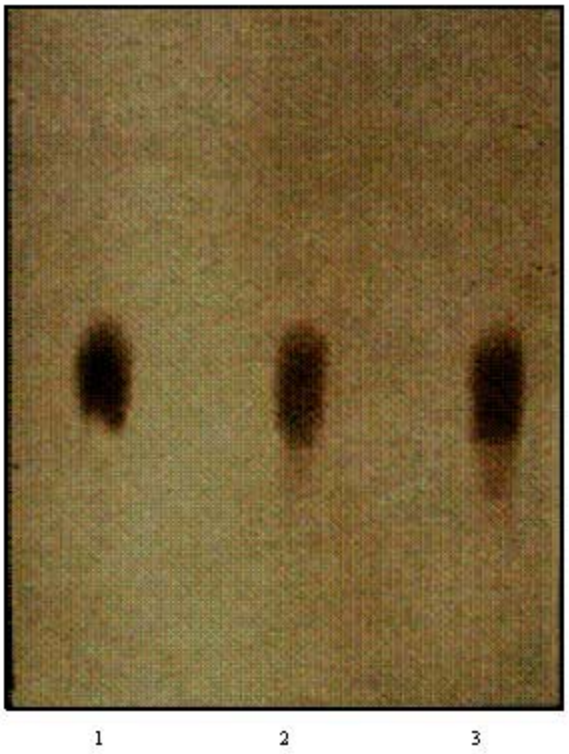

Fig. 2: Thin layer chromatogram of standard desferrioxamine (lane 1) and desferrioxamine produced by *S. griseoflavus* S7011 (lane 2) and C7031 (lane 3)

The most harmless and efficient time for protoplast to be exposed to PEG, has been suggested as 30-60 sec (Anne *et al.*, 1990; Baltz and Matsushima, 1981; Matsushima and Baltz, 1986). Therefore, protoplasts were treated with 50% PEG 1000 and after 1 min, it was diluted with an appropriate solution (Matsushima and Baltz, 1986) then mixed protoplasts were distributed on double-selective (containing sodium azide and crystal violet) and non-selective regeneration plates. Once incubation period expired, totally 58 colonies were appeared on all 10 regeneration plates (5.8 colonies per plate as average), while the average of colony number on non-selective plates was 132. Among 58 recombinant colonies, only 17 colonies (1.7 colonies per plate) could grow on double selective media. These 17 fused colonies (PF1-PF17) showed a diversity of desferrioxamine production, amongst them, three fused cells had almost similar production rates to C7031, four were like S7011, four were like *S. griseoflavus*, five had higher rates than three parental strains and 1 lost its ability of desferrioxamine production. Out of 5 fused cells, PF10 and PF9 showed increase in desferrioxamine production rate by 81.8% and 68.3% as compared to *S. griseoflavus*. In conclusion, the fused cells of *S. griseoflavus* PF10 was selected as hyper desferrioxamine producer and the work is in progress to drive another *S. griseoflavus* that produces desferrioxamine in high quantity.

REFERENCES

Alia, D., B. Marie-Noëlle, P. Jean-Pierr and E. Dominique, 1998. Dual role of desferrioxamine in *Erwinia amylovora* pathogenicity. Mol. Plant-Microbial Interaction, 11 (8): 734-742.

Anne, J., L. VanMelleart and H. Eyssen, 1990. Optimal conditions for efficient transformation of *S. venezuealae* protoplast. Applied Microbiol. Biotechnol., 32: 431-435.

Baltz, R.H. and P. Matsushima, 1981. Protoplast fusion in Streptomyces: Conditions for efficient genetic recombination and cell regeneration. J. Gen. Microbiol., 127: 137-146.

Francisco, J.F., R. Javier and F.M. Juan, 2003. Characterization of the iron-regulated desA promoter of *Streptomyces pilosus* as a system for controlled gene expression in *actinomycetes*. Microbial. Cell Factories, 2 (1): 5-14.

Hopwood, D.A. and K.F. Chater, 1984. Streptomycetes. In: Genetic and Breeding of Industrial Microorganisms, Ball, C. (Ed.). CRC Press, pp: 8-40.

Kazuki, Y., O. Hiroaki, O. Hiro-Omi, H. Kuniaki, S. Funnie, T. Hideaki, S. Shohei, B. Teruhiko and U. Kenji, 2005. Desferrioxamine E produced by *Streptomyces griseus* stimulate growth and development of *Streptomyces tanashiensis*. Microbiology, 151: 28899-28905.

Matsushima, P. and R.H. Baltz, 1986. Protoplast Fusion. In: Manual of Industrial Microbiology and Biotechnology, Demain, A.L. and N.A. Solomon (Eds.). American Society for Microbiology, Washington DC., pp: 170-183.

Paul, I., 2006. A commercially available iron chelating agent, desferrioxamine Fe and Al-mobilization in soil. Soil Biol. Biochem., 38 (6): 1491-1493.

Petter, H.H., 1985. Industrial Aspects of Iron Chelators: Pharmaceutical Applications. In: Protein of Iron Storage and Transport, Spik, G., J. Montreuil, R. Chrichton and J. Mazurier (Eds.). Amesterdam, Elsevier, pp: 293-303.

Siebner, H.F., Y. Hadar and Y. Chen, 2004. Interaction of iron chelating agents with clay minerals. Soil Sci. Soc. Am. J., 68: 470-480.

Silvia, K.R., J. Günther, N.R. Kenneth, M. Johannse and Z. Hans, 1992 Solution thermodynamic of ferric complexes of new desferrioxamine siderophores obtained by directed fermentation. J. Am. Chem. Soc., 114: 2224-2230.

Victor, R.G., E.T. Philip, M.B. Gary, Z. Stenford, M. Abraham, G. Flesch and P. Dean, 1992. Iron chelation with desferrioxamine B in adults with asymptomatic *Plasmodium falciparum parasitemia*. Blood, 79 (2): 308-312.