Technology Section

Pak j. sci. ind res., vol. 37, no.9, September 1994

PRODUCTION OF OXYTETRACYCLINE BY DIFFERENT MUTANTS OF *STREPTOMYCES RIMOSUS* INDUCED BY DRY AND WET HEAT IN A DATE MEDIUM

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(Received April 29, 1993; revised January 1, 1994)

Dry heat was more effective as physical mutagen than wet heat for *Streptomyces rimosus*. The optimal dose of temperature for inducing auxotrophic mutants in *Streptomyces rimosus* was 55° under dry treatment conditions. Two mutants only out of 138 dry and wet heat mutants produced higher antibiotic titres than the parent organism.

Key words: Oxytetracycline, Mutants of *S. rimosus*, Dry and wet heat, Date medium.

Introduction

Use of mutation as a tool for yield improvement is more likely to be immediately productive during the early years of development of an antibiotic than at a later date [1-13].

Heat treatment has not been used to induce mutation of *Streptomyces rimosus*, therefore, the objective of this research was to evaluate efficiencies of wet and dry heat treatment of the parent oxytetracycline producer.

Materials and Methods

Mutant strains. Auxotrophic mutants of *S. rimosus* induced by various mutagenic agents were detected on minimal medium isolated and maintained on slants of complete and sporulation media.

Detection and isolation of auxotrophic mutants. This was done by a total isolation procedure. A random sample of spores which survived each mutagen treatment was inoculated on CM templates (37 plates) and incubated for 3 days at 30°. Each template plate was in turn replicated on MM plates in duplicates and incubated at 30° for 4 to 5 days in the dark. All colonies which failed to grow on MM at the end of incubation period or grew very weakly were considered auxotrophic mutants and isolated from the original CM templates on CMor SM slants.

Sporulation medium (SM). The sporulation medium contained the following ingredients (g/1): sucrose 3.0, dextrin 15.0, urea 0.1, peptone 5.0, beaf extract 1.0, K_2HPO_4 0.5, $MgSO_4.7H_2O$ 0.5, NaCl 0.5, $FeSO_4.7H_2O$ 0.1 and agar 30 in 1000 ml distilled water.

Complete medium (CM). The complete medium contained the following ingredients (g/1): glucose 10.0, yeast extract 3.0, peptone 2.0, hydrolysed casein 2.0, $MgSO_4.7H_2O$ 0.5 and agar 20.0 in 1000 ml distilled water.

Minimal medium (MM). The minimal medium contained the following ingredients (g/l): glucose 10.0, asparagine 0.5, KH_2PO_4 0.5, KOH 0.3, $FeSO_4.7H_2O$ 0.05 and agar 20.0 in 1000 ml distilled water.

Replication of cultures. Cultures to be replicated were inoculated at 37 loci on a template petridish of CM. The loci were arranged in a 6x6 square with the 37th locus being at one side for the orientation of the replicates. This pattern allowed the 37 colonies to be replicated simultaneously by a 37-point replicator. Sterilization of the replicator needles was carried out by heating with a bunzen and they were cooled by stabbing into the sterile agar medium before charging them with inoculum for replication.

Mutagenic treatments. Spore suspension of *S. rimosus* was exposed to the physical mutagenic agent. In each treatment, spore suspension was prepared by washing slant culture on SM with sterilized tris buffer and well agitated on a shaker for 15 mins and filtered by exposing the spore suspension to the mutagenic agents in the following ways:

Heat mutagenesis. Since heat as a mutagenic agent received a little attention, one has to try different doses of heat under various circumstances to find out the most suitable experimental conditions for inducing auxotrophic mutants in *S. rimosus*. Therefore, different degrees of temperature higher than the optimal growth temperature for *S. rimosus* (30°) were used. The spores were suspended in solutions of tris buffer of 40°, 45°, 50°, 55° and 60° and immediately immersed in water baths having the same temperatures. A one ml samples were immediately diluted in 9 ml sterile water to serve as untreated control and serially diluted in sterile distilled water to reach a final dilution, for each sample, of 100 spores per millilitre. Subsequent samples were taken at regular intervals for each temperature as indicated in the results section. Serial dilutions of these samples in sterile water were made to reach a final

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dilution, for each sample, of 100 spores per millilitre. Samples of the final dilutions were spread on CM plates and subjected to the same method of determining the percentage of survival in the previous experiments.

In another set of experiments, the spores of *S. rimosus* were exposed directly to the same temperatures without being suspended in tris buffer to avoid any effect of wet environment as reported by Barnes [11] who was not able to induce mutation in *Eurotium herbariorum* when the spores were suspended in water but when exposing the spores to heat in dry chambers he obtained mutants. Therefore, cultures of *S. rimosus* on plates of SM were exposed directly to 40°, 45°, 50°, 55°and 60° in incubators, each of them was adjusted to one of these temperatures and spore samples were taken immediately at the beginning of each treatment and then at regular intervals as in the previous set of treatments and subjected to the same process of determining the percentage of survival as in the previous experiments.

Maintenance medium. Different mutants produced when *streptomyces rimosus* NRRL B-2234 was treated by dry and wet heat were maintained on the medium containing the following ingredients (g/1): glucose, 10.0; peptone, 2.5; yeast extract, 2.5; KH_2PO_4, 1.0; $MgSO_4.7H_2O$, 0.25; $MnSO_4.4H_2O$, 0.025; $FeSO_4.7H_2O$, 0.005 and agar, 30.0, in 1000 ml distilled water.

Vegetative medium. The vegetative medium used for growing the different mutants of *Streptomyces rimosus* contained the following ingredients (g/1): glucose, 10.0; peptone, 5.0; yeast extract, 5.0; KH_2PO_4, 1.0; $MgSO_4.7H_2O$, 0.25; $MnSO_4.4H_2O$, 0.025 and $FeSO_4.7H_2O$, 0.005; in 1000 ml distilled water.

Fermentation medium. Different mutants produced by dry and wet heat treatment were grown fermentatively in the fermentation medium to determine their potencies for the formation of oxytetracycline. The fermentation medium contained the following ingredients (g/1): date-coat sugar extract, 20.0; date-seed hydrolysate, 10.0; urea, 1.5; KH_2PO_4, 1.0; date-seed lipid, 1.0 and date-seed ash, 0.5; in 1000 ml distilled water. The ingredients of the fermentation medium were thoroughly mixed and the initial pH of the medium was adjusted to 6.0. The medium was portioned into Erlenmeyer flasks (capacities, 250 ml), each flask contained 50 ml. The flasks were plugged with cotton and sterilized at 121° for 20 min. When the flasks attained room temperature, they were inoculated under aseptic conditions with a standard inoculum of the mutants of *Streptomyces rimosus.* The percentage of inoculum was 1.0. The inoculated flasks were inserted in shaker (200 rpm) at 30° for 144 hrs. At the end of the fermentation process, the flasks were taken and harvested to measure the final pH value of the fermented medium, residual sugars, cell biomass of the experimental mutant and oxytetracycline formed in the fermented medium.

Microbiological determination of oxytetracycline. Oxytetracycline produced in the fermented media by different mutants was determined by the method of Abou-Zeid and Shehata [14] and Kavanagh [15].

Determination of residual sugars. Sugars were determined spectrophotometrically by the method of Somogyi [16].

Determination of pH. The initial and final pH values of the fermentation medium were measured using a Corning Scientific Instrument, Model 12, Research pH-meter.

Determination of microbial cell biomasses. Cell biomasses of different *Streptomyces rimosus* mutants were determined at the end of fermentation process. The fermented medium was centrifuged at 4000 rpm for 20 mins to cediment the microbial cells of *Streptomyces rimosus* mutants. The microbial cells were dried at 95° to constant weight.

Results and Discussion

When the effects of different temperatures for different exposure times on the percentages of survival and auxotrophic mutants were studied, it was taken into consideration that the duration of exposure to each temperature would be different from one temperature to another. The spores were exposed to 40° for 5 hrs., to 45° for 4 hrs., to 50° for 3 hrs., to 55° for one hr. and to 60° for 30 mins and samples were taken at regular intervals for each temperature. In other words, there was a gradual decrease in the total exposure time at higher temperatures. This was done as an attempt to find out the optimal temperature dose that gives the highest possible mutation percentage with the highest possible survival percentage.

The results show that the percentage of survival decreased as time of exposure for each temperature increased whereas the percentage of mutation increased as confirmed by linear regression comuptation. Comparing the total percentage of auxotrophic mutants in the wet and dry treatments for each temperature (Table 1), the total percentage of auxotrophic mutants resulted from a dry treatment was almost twice than the total percentage of auxotrophic mutants resulted from a wet treatment. The opposite would hold true for total percentage of survival but the difference between wet and dry treatments was about within the range from 5 to 20%. The highest total percentage of auxotrophic mutants was induced by temperature 55° in the dry treatment which was 3.06%. Therefore, the optimal dose of temperature for inducing auxotrophic mutants in *S. rimosus* would be 55° under dry treatment conditions. It might be possible to measure mutation percentage at this temperature by using longer exposure time. Future study in this respect would be revealing. Our results

TABLE 1. TOTAL SURVIVAL AND RECOVERY OF AUXOTROPHIC MUTANTS RESULTING FROM EXPOSURE OF SPORES OF *S. RIMOSUS* TO DIFFERENT TEMPERATURES.

Temperature (°C)	Mode of exposure	Total exposure time (min)	Total* survivors (%)	Auxotrophic mutants		
				No. of colonies tested	No. of mutants	Percentage
40	Wet	300	56.4	1782	8	0.45
	Dry	300	89.0	867	14	1.61
45	Wet	240	88.90	1036	19	1.83
	Dry	240	79.15	1036	31	2.99
50	Wet	180	43.80	1646	9	0.54
	Dry	180	39.53	2953	20	0.67
55	Wet	60	55.3	1089	16	1.47
	Dry	60	52.3	1143	35	3.06
60	Wet	30	63.2	925	8	0.86
	Dry	30	61.3	925	16	1.72

$$\text{* Total survivors} = \frac{\text{Total No. of survivors x 100}}{\text{Total No. of treated spores}}$$

showed the possibility of inducing mutants under wet conditions which is contrary to what was reported by Barnes [10]. This could be explained as species difference since each species responds differently to each mutagenic agent.

Potencies of the different mutants of S. rimosus induced by dry heat. The antibiotic yields produced by mutants of *S. rimosus* induced by dry heat (40°, 50°, 55° and 60°) were less than antibiotic yield of the parent organism. Only two mutants M-12 and M-22 (Table 2) out of 20 mutants induced by dry heat (45°) gave high titres for oxytetracycline than the parent organism.

Efficacies of the different mutants of S. rimosus induced by wet heat. The antibiotic yields produced by mutants of *S. rimosus* induced by wet heat (40°, 45°, 50°, 55° and 60°) were less than the parent organism.

Surveying potencies of the different mutants induced by dry and wet heat at 40°, 45°, 50°, 55° and 60° for the formation of oxytetracycline were carried out. The data showed that the mutants could be divided into three categories. The first category included active mutants which have low titres of the antibiotic than the initial micro-organism. The second category included non-oxytetracycline producers. The third category included only two mutants (M-12 and M-22) which have higher titres for the antibiotic than the initial organism.

Acknowledgement. The authors express their gratitude to King Abdulaziz City for Science and Technology (KACST) for the financial support of this work (AR-9-15).

TABLE 2. POTENCIES OF THE DIFFERENT MUTANTS OF *S. RIMOSUS* INDUCED BY DRY HEAT (45°) FOR THE FORMATION OF OXYTETRACYCLINE (OTC).

Time of exposure (mins)	Mutants	Final pH values*	Residual sugars (mg/ml)	Microbial biomass (mg/ml)	OTC (µg/ml)
80	M-1	5.7	5.8	10.9	0
80	M-2	5.8	6.6	8.5	310
80	M-3	5.7	6.6	7.1	340
80	M-4	6.6	6.9	10.6	0
80	M-5	6.1	2.3	8.3	0
160	M-6	6.2	6.5	9.0	0
160	M-7	6.3	6.2	7.8	310
160	M-8	5.7	6.0	10.1	0
160	M-9	6.4	8.2	5.0	0
160	M-10	6.3	6.1	8.9	307
160	M-11	6.4	5.6	9.4	360
160	M-12	6.4	7.3	6.5	660
160	M-13	6.3	8.4	8.1	310
160	M-14	6.4	7.6	5.2	360
160	M-15	6.2	8.8	3.6	100
160	M-16	6.1	8.3	5.2	107
160	M-17	5.6	7.5	4.0	100
160	M-18	6.3	7.1	6.6	326
160	M-19	6.8	9.2	5.2	305
160	M-20	5.5	7.9	3.9	100
160	M-21	6.7	6.6	5.7	0
160	M-22	4.6	6.1	6.7	663
160	M-23	7.6	7.3	5.1	204
240	M-24	6.6	7.3	5.1	204
240	M-25	7.6	8.5	3.0	0
240	M-26	6.9	6.6	4.9	0
240	M-27	7.2	7.1	4.4	0
240	M-28	5.9	6.1	4.7	0
240	M-29	6.3	5.2	4.4	126

* The initial pH value of the fermentation medium was 6.0.

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