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PRODUCTION OF OXYTETRACYCLINE BY DIFFERENT MUTANTS OF *STREPTOMYCES RIMOSUS* INDUCED BY PHYSICAL MUTAGENS IN A DATE MEDIUM

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Sixty seven mutants produced by different physical mutagens were surveyed for antibiotic formation. One mutant out of 18 UV-mutants produced higher antibiotic titres than the initial organism, while in case of the 49 x-rays mutants, none of the mutants produced antibiotic yield more than the parent organism. UV irradiation gave the highest total auxotrophic mutants percentage.

Key words: UV, X-ray, Mutagens-oxytetracycline

Introduction

Oxytetracycline is produced by different species of *Streptomyces rimosus, Streptomyces henetus, Streptomyces platensis*, and *Streptomyces capuensis* [1-4].

The production of high yield strains from *S. rimosus* has been accomplished by induced mutations caused by X-ray, UV irradiation and nitrogen mustard [5-7]. The best results were obtained by UV irradiation. Bornstajn and Wolf [8] produced high yield strains by treating *S.rimosus* with UV radiation and methyl - bis - B - chlorethylamine. Mindlin *et al.* [9] reported that the genetic control of antibiotic bio-synthesis of oxytetracycline was obtained by *S.rimosus*. The highly active strain LST-118 which was changed by various mutagen (U.V. irradiation, ethylenimine, diethyl-sulphate), the 70 mutants obtained were divided into two groups according to their phenotypic analysis. The amount of oxytetracycline produced was within broad limits (from 100 to 1200 µg/ml).

In order to exploit local natural renewable resources, Saudi date components, especially the fleshy part (epi-and mesocarp) of the tamer stage was utilized in the fermentation medium for the formation of oxytetracycline.

The objective of this research was to treat the parent oxytetracycline producer with UV light and X-rays. The mutants thus produced were used on the medium containing Barni date fleshy part water extract for the formation of oxytetracycline to evaluate the efficiencies of these mutants.

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 carried out by a total isolation procedure. A random sample of spores which survived each mutagen treatment was inoculated on CM templates (37/plate) and incubated for 3 days at 30°C. Each template plate was in turn replicated on MM plates in duplicates and incubated at 30°C 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 CM/ or SM slants.

Sporulation medium (SM). The sporulation medium contained the following ingredients (g/l): 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, $FeSO_4.7H_2O$ 0.01, agar 30; in 1000 ml of distilled water.

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

Replication of cultures. Cultures to be replicated were inoculated at 37 loci on a template petri-dish 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 them with a bunzen burner and they were cooled by stabbing them into sterile agar medium before charging them with inoculum for replication.

Mutagenic treatments. Spore suspensions of *S. rimosus* were exposed to different physical mutagenic agents. In each treatment spore suspension was prepared by washing slant cultures on SM with sterilized tris buffer and well agitating on a shaker for 15 min and filtered by exposing the spore suspension to the mutagenic agents in the following ways:

UV light mutagenesis. Astralux disinfection (germicidal) lamp. 30 watts, 254nm wave length, model EKL 30H was used as the source of UV light. The distance between the spore suspension and the lamp was 30 cm. Before exposing the spore suspension, a one ml sample of it was diluted in 9 ml sterile water to serve as untreated control and serially diluted in sterile distilled water to halt the mutagenic treatment. The spore suspension was immediately exposed to the UV light and subsequent samples were taken at regular intervals (30, 60, 90 and 120 sec). Samples of the final dilutions were then subjected to determine the percent of survivals.

X-rays mutagenesis. The spore suspension was exposed to 30 kilorads of X-ray (Linear Accelerator type, a MV). Choice of X-rays dose was based in the work of Mandal *et al.* [10]. A one ml sample was immediately diluted in 9 ml sterile water to serve as control and serially diluted in sterile distilled water to reach a final dilution of 100 spores per milli litre. Subsequent samples were taken at regular intervals (30, 60, 90 and 120 min). Samples of the final dilutions were then subjected to determine the percent of survivals.

Maintenance medium. The different mutants produced when *Streptomyces rimosus* NRRL B-2234 was treated by the different physical mutagenic agents were maintained on the medium containing the following ingredients (g/l): 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, agar 30.0, in 1000 ml distilled water.

Vegetative medium. The vegetative medium used for growing the different mutants of *S. rimosus* contained the following ingredients (g/l): glucose 10.0, peptone 5.0, yeast extract 5.0, KH_2PO_4 1.0, $MgSO_4.7H_2O$ 0.25, $MnSO_4$ 0.025, $FeSO_4.7H_2O$ 0.005, in 1000 ml distilled water. The ingredients of the vegetative medium were thoroughly mixed and the initial pH value of the medium was adjusted to 6.0.

Fermentation medium. The different mutants produced by the different physical mutagenic techniques were fermentatively grown in the fermentation medium to determine their potencies for the formation of oxytetracycline. The fermentation medium contained the following ingredients (g/l): date-coat sugar extract 20.0, date seed hydrolysate 10.0, urea 1.5, KH_2PO_4 1.0, date-seed lipid 1.0, dated 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°C for 20 min. When the flasks attained from temperature, they were inoculated under aseptic conditions with a standard inoculum of the mutant of *S.rimosus*. The percentage of inoculum was 1.0. The inoculated flasks were inserted in a shaker (200 rpm) at 30°C for 144 hr. At the end of the fermentation process, the flasks were taken and harvested to measure the following parameters:

Final pH value of the fermented medium, residual sugars, cell biomass of the experimental mutant, oxytetracycline formed in the fermented medium.

Microbiological determination of oxytetracycline. Oxytetracycline produced in the fermented media by the different mutants was determined by the method of Abou-Zeid and Shehata [11] and Kavanagh [12].

Determination of residual sugars. Sugars were determined spectrophotometrically by the method of Somogyi[13].

Determination of pH. The initial and final pH values of the fermentation medium were carried out by pH-meter (Corning Scientific Instruments, Model 12, Research pH-meter).

Determination of the microbial cell biomasses. Cell biomasses of the different *S. rimosus* mutants were determined at the end of fermentation process. The fermented medium was centrifuged at 4000 rpm for 20 min to precipitate the microbial cells of *S. rimosus* mutants. The microbial cells were dried at 95°C till constant weights.

Results and Discussion

UV light mutagenesis. The results of treating the spores of *S. rimosus* with UV light for different exposure times and its effect on survival and auxotrophic mutants percentages are shown in Table 1. The percentage of survival decreased lineary as time of exposure increased whereas the percentage of auxotrophic mutants increased lineary as time of exposure increased as confirmed by linear regression computation.

TABLE 1. SURVIVAL AND RECOVERY OF AUXOTROPHIC MUTANTS RESULTED FROM UV LIGHT-TREATED SPORES OF *S. RIMOSUS*.

Exposure time (sec)	Survivors (%)	Number of colonies tested	Auxotrophic mutants	
			No.	%
0	100	53	0	0.0
30	22.64	12	6	50.0
60	18.88	10	2	20.0
90	20.75	11	2	18.2
120	26.42	14	8	57.1
Total	37.70*	100	18	18.0

*Total survivor % = $\frac{\text{Total No. of survivors x 100}}{\text{Total No. of treated spores}} = \frac{10000}{265} = 37.70\%$

Table 1 shows that the total survivor percentage is 37.7% and the total auxotrophic mutant percentage is 18%. El-Adawy [14] reported a higher survival percentage than ours and a lower mutant percentage than ours. This was expected since the dose of UV light used in this work was higher than the one used by El-Adawy [14] and a longer exposure time was used in this work.

X-rays mutagenesis. Survival and recovery of auxotrophic mutants upon treating the spores of *S. rimosus* with X-rays for different exposure times are presented in Table 2. The same relation holds true between X-rays and each of survival and mutation percentages was in the previous results. The total survivors percentage was 62.1% and that of auxotrophic mutants was 8.11%. X-rays gave in this respect a higher total percentage of survival than UV light but gave a lower total percentage of auxotrophic mutants than UV light. There are no available data in the literature to see whether this is always the case whenever auxotrophic mutants or another kinds of mutants were induced by X-rays and UV light.

Potencies of the different mutants of *S. rimosus* induced by UV irradiation for the formation of oxytetracycline.

The data obtained Table 3 show that when the parent organisms was exposed to UV irradiation for different periods (30, 60, 90 and 120 min), 18 mutants were produced.

The 18 mutants were cultivated in the date-medium to evaluate their potentialities for the fermentative formation of oxytetracycline and other parameters such as the final pH value of the fermented medium, residual sugars and the microbial biomass were also determined.

The initial pH value of the fermentation medium was adjusted to 6.0, and at the end of the fermentation process, it was measured. The final pH value of the fermented medium was shifted to acid side and this may be correlated to the formation of organic acids and acidic amino acids. The final pH values were different depending upon the activities of each specific mutant.

TABLE 2. SURVIVAL AND RECOVERY OF AUXOTROPHIC MUTANTS RESULTED FROM X-RAYS TREATED SPORES OF *S. RIMOSUS.*

Exposure time (Min)	Survivors (%)	Number of colonies tested	Auxotrophic mutants	
			No.	%
0	100	150	0	0.0
30	85	150	10	6.67
60	71.6	150	9	6.00
90	32.3	97	14	14.43
120	21.6	65	16	24.62
Total	62.1*	612	49	8.11

$$* \text{ Total survivors } \% = \frac{\text{Total No. of survivors x 100}}{\text{Total No. of treated spores}} = \frac{93100}{1500} = 62.1\%$$

The 18 mutants utilized the date sugars, but their utilization were also different depending upon the specific mutants. Different microbial biomasses were also obtained depending upon the activity of each specific mutant.

With respect to the antibiotic formation, the 18 mutants produced different amounts of oxytetracycline. The 18 mutants could be classified into three groups. The first group included 14 active mutants producing oxytetracycline, but their potentialities for the antibiotic formation were less than the initial organism. The second group included 3 inactive non-producing oxytetracycline mutants and the third group included one active mutant, which produced high titres for the antibiotic more than the initial organism. The most active mutant was M-14. The percentage of increase in the antibiotic yield of M-14 more than the parent organism was about 77.

The highly active strain LST-118 which was changed by various mutagens (UV light, ethylenimine, diethyl sulfate), the 70 mutants obtained were divided into 2 groups according to their phenotypic analysis. The amount of oxytetracycline produced was within broad limits (from 100 to 1200 µg/ml).

Potencies of the different mutants of *S. rimosus* induced by X-rays irradiation for the formation of oxytetracycline.

The data obtained (Table 4) show that when the initial microorganism was exposed to X-rays irradiation for the

TABLE 3. POTENCIES OF THE DIFFERENT MUTANTS OF *S. RIMOSUS* INDUCED BY UV IRRADIATION FOR THE FORMATION OF OXYTETRACYCLINE (OTC)

Time (Sec.)	Mutants	Final pH Value*	Residual sugars (mg/ml)	Microbial biomass (mg/ml)	OTC (µg/ml)
30	M-1	4.3	7.5	6.5	178
30	M-2	4.2	20.0	0.8	0
30	M-3	8.0	21.0	0.4	0
30	M-4	10.0	10.0	5.1	130
30	M-5	4.1	10.4	7.2	112
30	M-6	4.1	11.7	5.5	143
60	M-7	4.5	6.6	4.7	887
60	M-8	4.5	6.9	5.7	563
90	M-9	4.1	10.0	7.6	126
90	M-10	3.3	10.8	3.6	0
120	M-11	4.2	4.4	4.1	170
120	M-12	4.2	4.2	6.6	150
120	M-13	4.7	7.8	6.5	250
120	M-14	4.3	6.8	6.0	972
120	M-15	4.4	6.9	7.3	110
120	M-16	4.1	9.6	6.4	504
120	M-17	4.2	7.6	7.6	334
120	M-18	4.1	7.7	6.8	235

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

Table 4. Capabilities of the Different Mutants of *S. rimosus* Induced by X-rays Irradiation for the Formation of Oxytetracycline (OTC).

Time (Min) (ug/ml)	Mutants	Final pH value*	Residual sugars	Microbial biomass (mg/ml)	OTC (mg/ml)
30	M-1	5.2	3.1	8.3	334
30	M-2	6.0	3.5	8.0	163
30	M-3	5.9	1.6	7.6	0
30	M-4	4.5	3.1	4.5	197
30	M-5	4.2	1.6	6.1	178
30	M-6	6.0	2.3	6.8	334
30	M-7	5.2	0.6	5.5	0
30	M-8	6.7	1.3	2.0	0
30	M-9	4.5	1.6	5.0	163
30	M-10	4.4	4.0	7.5	197
60	M-11	5.1	1.8	5.9	0
60	M-12	4.1	1.1	8.8	220
60	M-13	5.1	2.8	12.6	209
60	M-14	4.2	1.7	8.0	197
60	M-15	4.3	2.5	9.2	0
60	M-16	4.4	4.3	8.0	163
60	M-17	–	–	–	–
60	M-18	5.3	2.3	8.4	163
60	M-19	4.1	2.2	5.8	197
90	M-20	4.3	3.8	7.8	205
90	M-21	4.3	4.5	7.8	105
90	M-22	4.4	1.2	6.9	140
90	M-23	6.6	1.8	8.7	390
90	M-24	4.5	1.0	9.2	0
90	M-25	4.1	1.2	4.3	197
90	M-26	4.4	1.9	8.9	187
90	M-27	4.3	3.9	10.5	140
90	M-28	5.4	3.6	8.8	250
90	M-29	5.5	2.1	8.9	0
90	M-30	4.6	3.5	5.2	197
90	M-31	5.9	5.7	5.5	0
90	M-32	4.4	2.4	10.3	178
90	M-33	4.2	2.0	8.7	220
120	M-34	6.5	3.7	10.2	280
120	M-35	5.7	2.0	7.6	460
120	M-36	5.7	3.1	8.8	425
120	M-37	4.5	5.6	8.6	104
120	M-38	4.0	1.4	7.6	197
120	M-39	5.8	10.0	6.9	0
120	M-40	5.1	2.7	6.4	197
120	M-41	3.6	2.9	5.0	150
120	M-42	4.5	1.1	7.9	0
120	M-43	4.0	1.2	7.2	197
120	M-44	4.5	5.6	9.8	178
120	M-45	6.2	2.0	8.6	550
120	M-46	4.1	4.5	10.5	150
120	M-47	4.3	2.6	8.8	163
120	M-48	4.1	3.2	4.5	197
120	M-49	3.9	1.8	8.3	197

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

different periods (30, 60, 90 and 120 min), 49 mutants were obtained.

The 49 mutants were fermentatively cultivated in the date medium to evaluate their potencies for the biosynthetic formation of oxytetracycline and other parameters such as the final pH value of the fermented medium, residual sugars, and the microbial biomass were also determined.

The initial pH value of the fermentation medium was adjusted to 6.0 and at the end of the fermentation process, it was shifted towards more acid side in most of the mutants investigated. The final pH value ranged from 3.6 to 6.7 depending upon the activities of each specific mutant. The 49 mutants utilized the date sugars, but their rates of utilization were also different depending upon the metabolic activities of each specific mutant.

Concerning the antibiotic formation, most of the mutants produced different amounts of the antibiotic. The 49 mutants could be classified into three groups. The first group included 38 mutants producing oxytetracycline, but their productivities for the antibiotic formation were less than the initial organism. The second group included 10 mutants which were inactive non-oxytetracycline producers. The third group included one mutant (M-45) which gave the same antibiotic titre similar to the initial microorganism.

The 67 mutants (18 UV-mutants, 49 X-rays mutant) exhibited variations in their potentials for the antibiotic formation. Out of 18 UV-mutants, 2 mutants (M-7 and M-14) gave high antibiotic titres than the initial organism. The X-rays mutants did not give rise to mutants capable of antibiotic yield more higher than the parent strains.

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