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THE FERMENTATIVE PRODUCTION OF OXYTETRACYCLINE BY STREPTOMYCES RIMOSUS*

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Abstract. Three strains of *Streptomyces rimosus* were grown on four different media. Medium I was suitable for the production of oxytetracycline by *Streptomyces rimosus* 12907) (Institute For Fermentation, Japan). The carbon and nitrogen sources of medium I were replaced by black strap molasses, fodder yeast (40% total protein) and rice bran. Suitable concentrations of molasses, fodder yeast and rice bran were 30.0, 20.0 and 10.0 g/l respectively. The medium also contained (g/l): KH_2PO_4 0.2 and $CaCO_3$ 1.0. The fermenter (capacity 1200 litre) was aerated by sterile air obtained from a specially designed system, the fermented medium (700 litre) extracted with n-butanol yielded 850.09 crude oxytetracycline.

Tetracyclines are closely related compounds widely used in the treatment of infectious diseases. Chlortetracycline was isolated by Dugger.[1] Finlay *et al.*[2] discovered oxytetracycline. Later, Stephens *et al.*[3] demonstrated that chlrotetracycline and oxytetracycline have the same general structure with a common basal nucleus which is responsible for their antibiotic activities.

Streptomyces species producing these antibiotics are *Streptomyces aureofaciens*, *Streptomyces rimosus*, *Streptomyces fuscofaciens* and *Streptomyces feofaciens*. The production of the antibiotic substances is associated with a certain stage of development of the organisum and is determined by the culture conditions.[4-8]

The carbon and nitrogen sources play an important role in the biosynthesis of tetracyclines by *Streptomyces* species.[9-24] The tetracycline antibiotics display features indicative of an acetate origin.

The economic potential of the production of such antibiotics depends on the availability of cheap substances. In Egypt, there are raw byproducts of many industries such as black strap molasses, fodder yeast, rice bran and corn bran which can be used in the fermentation processes.

The present work deals with the fermentative production of oxytetracycline by *Streptomyces rimosus* using raw byproducts and materials as constituents of the production medium.

Experimental

Strains of Streptomyces rimosus. Active strains of *Streptomyces rimosus* 12907 (Institute for Fermentation, Juso-Nishino-Cho, Higashiyodogawa,-Ku, Osaka, Japan), *Streptomyces rimosus* Finlay *et al.* 93060 and *Streptomyces rimosus* NRRL-2234 were used in the fermentative production of oxytetracycline. The microorganisms were maintained on the medium containing the following ingredients (g/l): glucose 10.0, peptone 5.0, KH_2PO_4 1.0, $MgSO_4.7H_2O$ 0.5, agar agar 20.0, distilled water to make 1000 ml. The initial pH of the medium was adjusted to 6.0-6.5. The total ingredients were thoroughly mixed and portioned into test tubes. The tubes were plugged with cotton wool and sterilized at 120°C for 20 min. When the test tubes containing the maintaining medium attained room temperature they were inoculated with the different strains of *Streptomyces rimosus*. The inoculated slants were incubated at 30° for 10 days to obtain luxuriant growth and sporulation.

Vegetative Medium. The active strains of *Streptomyces rimosus* were grown on the vegetative medium containing the following ingredients (g/l): glucose 10.0, peptone 5.0, KH_2PO_4 1.0, $MgSO_4.7H_2O$ 0.5 and distilled water to 1000 ml. The initial pH of the vegetative medium was adjusted to 7.0. The medium was portioned into 250-ml Erlenmeyer flasks each containing 50 ml and the flasks were then plugged with cotton wool and sterilized at 120°C for 20 min. When the flasks attained room temperature, they were inoculated with a standard spore suspension of *Streptomyces rimosus* under aspetic conditions. The inoculated flasks were inserted on a rotary shakes (200 rev/min) at 27°C for 48 hr. The vegetative medium was used for the inoculation of the fermentation medium.

Fermentation Medium. The fermentation media used for the fermentative production of oxytetracycline by *Streptomyces rimosus* contained the following components (g/l): Medium 1. soybean meal 10.0, glucose 10.0, NaCl 5.0 and $CaCO_3$ 1.0; Medium 2. soybean meal 5.0, glucose 10.0, glycerol 2.5, NaCl 5.0 and $CaCO_3$ 1.0; Medium 3. peptone 5.0, glucose

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10.0 and molasses 20.0; Medium 4. soybean meal 10.0, corn steep liquor 20.0, dextrin 10.0, NaCl 5.0, $CaCO_3$ 2.0 and KH_2PO_4 2.0. The initial pH of the fermentation media was adjusted to 7.0.

The carbon source of the fermentation medium was replaced by different concentrations of black strap molasses to select the amount of molasses giving the best antibiotic yield. The organic nitrogen source of the suitable fermentation medium was also replaced by different concentrations of fodder yeast, or rice bran to determine the concentrations of these ingredients giving high titre of oxytetracycline. The fermention medium was portioned into 500-ml Erlenmeyer flasks each containing 100 ml. The flasks containing the fermentation medium were plugged with cotton wool and sterilized at 120°C for 20 min. When the flasks attained room temperature, they were inoculated with a standard inoculum of the vegetative medium containing the growing cells of *Streptomyces rimosus* under aseptic conditions. The percentage of inoculum was 5.0. The inoculated flasks were inserted on a rotary shaker (200 rev/min) at 27°C for different incubation periods. After 24 hr, three flasks were taken off and the final pH of the fermented medium, dry materials and amount of oxytetracycline produced were determined.

Treatment of Molasses. Black strap molasses of sugar industries contain variable constituents. Some ingredients are essential for the growth of *Strepomyces rimosus* and antibiotic production, others are inhibitors both for the microbial growth and formation of the required antibiotic. The unwanted ingredients are restricted in the muddy precipitates, therefore, molasses is diluted with distilled water. The diluted molasses is centrifuged and the supernatant liquor is used in the fermentation medium.

Biological Determination of Oxytetracycline. A biological standard curve was drawn between log of different concentrations of oxytetracycline and inhibition zones of the susceptable bacterium *Bacillus subtilis* NRRL B-543.[26]

Purification of Air. A system was designed to obtain sterile air used in the fermentative process of oxytetracycline. The air was compressed in a reservoir provided internally with a heater. The hot air (110°C) was passed through five columns, four columns were charged with activated charcoal, while the fifth one was left empty. Before passing the hot compressed air, the charcoal present in the columns was activated by superheated steam for 10 hr. The hot air was passed through the activated charcoal to remove the impurities suspended in air. The sterile air was introduced into the fermenter (1200 litre capacity).

Production of Oxytetracycline. The volume of the fermentation medium was scaled up to be used in the fermentative production of oxytetracycline in a fermenter (1200 l). The fermentation medium contained the following ingredients (g/l): molasses 30.0, fodder yeast 20.0 and KH_2PO_4 0.5. The intial pH of the fermentation medium was adjusted to 6.5. Before charging the fermenter, it was thoroughly washed and sterilized by steam. 700 l of the fermentation medium were introduced into the fermenter. The medium was sterilized at 120°C for 40 min. When the fermenter attained room temperature, it was inoculated with 5% by volume of *Streptomyces rimosus* grown on the vegetative medium mentioned above. The fermentation medium was supplemented with cottonseed oil as an antifoam (0.5%). The fermentation medium was stirred and supplemented with sterile air. The fermentation process was conducted at 27° for 96 hr. After every 24 hr a sample was taken to determine the pH of the fermented medium, suspended dry matters and antibiotic yield.

Extraction of Oxytetracycline. The whole broth of the fermented medium (700 l) was adjusted to pH 2.0 and filtered. The filtrate was readjusted to pH 9.0 and extracted with n-butanol. The n-butanol extract was concentrated at 50° and extracted with 0.1N HCl. The extract was neutralized to precipitate oxytetracycline. Oxytetracycline precipitated was dried in an oven under reduced pressure at 45-50° and assayed by biological evaluation.

Results

Production of Oxytetracycline. Four different media were used for the fermentative production of oxytetracycline by three strains of *Streptomyces rimosus* (*Streptomyces rimosus* 12907, *Streptomyces rimosus* Finaly *et al.* 93060 and *Streptomyces rimosus* NRRL-2234). The results indicated that variable amounts of oxytetracycline were obtained. *Streptomyces rimosus* 12907 of the Institute for Fermentation of Japan produced the greatest amounts of oxytetracycline on the four different media. The initial pH of the different media were 7.0-7.1 and at the end of the fermentation process, it was shifted towards alkaline side (8.0-8.5). Medium I and *Streptomyces rimosus* 12907 were selected for further studies on the fermentative production of oxytetracycline.

The fermentation process of oxytetracycline production required 96 hr. The amount of oxytetracycline produced by the microorganism increased with incubation period reaching its optimum at 96 hr, above which a decrease was obtained. A drop in the pH was obtained during 48-hr fermentation process and this may be correlated to the accumulation of organic acids which were further utilized by the microorganism for its different metabolic processes and at the end of the fermentation process, the final pH was pushed to alkaline side (8.5).

Replacement of Carbon and Nitrogen Sources of Medium 1. Medium 1 contained costly ingredients, therefore, certain local ingredients such as black strap molasses, fodder yeast and rice bran were used for the fermentative production of oxytetracycline by *Streptomyces rimosus.* The results obtained (Table 1) showed that when glucose of Medium 1 was replaced by different concentrations of black strap molasses, the amount of oxytetracycline produced increased with the increase of black strap molasses reaching its optimum at 30.0-40.0 g/l, above which a decline in the yield was obtained. The final pH was variable, depending on the amount of molasses added. Molasses (30.0 g/l) was used instead of glucose and soybean

meal was replaced by different concentrations of fodder yeast (40% total protein), while the other ingredients of the medium remained unchanged. The results showed that the amount of oxytetracycline produced increased with the increase of fodder yeast concentration reaching its optimum at 20.0 g/l, above which a decrease in the antibiotic yield was recorded. Substitution of fodder yeast for soybean meal improved the yield of the antibiotic. The final pH of the fermented medium was 7.5-8.5, depending on the amount of fodder yeast added to the production medium. Molasses (30.0 g/l) was used instead of glucose; and soybean meal was deleted and replaced by different concentrations of undefatted rice bran, while other ingredients of the medium remained unchanged. The amount of oxytetracycline excreted in the medium increased with the increase of rice bran concentration reaching its optimum at 30.0-40.0 g/l, above which a decline in the antibiotic yield was obtained. Different concentrations of undefatted rice bran in the presence of molasses (30.0 g/l) and fodder yeast (40% total protein) 20.0 were incorporated into the medium with deletion of soybean meal and removal of other constituents. The results obtained (Table 1) indicated that the addition of rice bran besides fodder yeast (40% total protein) favoured production of high antibiotic yield. The amount of oxytetracycline excreted in the medium increased with the increse of rice bran concentration reaching its optimum at 10.0 g/l, above which a decrease in the antibotic yield was obtained.

Molasses (30% g/l), rice bran (10%) and different concentrations of fodder yeast (40% total protein) were incorporated into the medium with deletion of soybean meal, while the other ingredients of the medium remained unchanged. The results obtained (Table 1) showed that suitable concentration of fodder yeast in presence of molasses and rice bran was 20.0 g/l. The amounts of molasses, fodder yeast and rice bran suitable for the antibiotic production were 30·0, 20.0 and 10% respectively. These amounts of ingredients were used in the fermentation medium besides NaCl and $CaCO_3$ and different concentrations of KH_2PO_4 were added. The results recorded (Table 1) showed that the amount of oxytetracycline produced increased with the increase of KH_2PO_4 concentration reaching its optimum at 0.2 g/l above which a decrease in the antibiotic yield was no ted. The addition of $MgSO_4$ to the fermentation medium did not push the antibiotic yield to higher titre. NaCl was deleted from the fermentation medium and the antibiotic yield was not affected.

Scaling Up of Suitable Ingredients. The above-mentioned ingredients which were good substances for the fermentative production of oxytetracycline, were scaled up in a fermenter (1200 l). The results indicated that the initial pH value of the fermentation medium was 7.0, at the end of the fermentation process it was directed to 8.5, while suspended dry matter was 12.5 mg/ml. At the end of the fermentation process, the whole volume (700 l) was adjusted to pH 2.0 and filtered. The filtrate was adjusted to pH 9.0 and thoroughly extracted with n-butanol. The amount of oxytetracycline was 0.850 g/l. Crude oxy-

TABLE 1. OXYTETRACYCLINE PRODUCED BY *Streytomyces rimosus* WHEN GROWN ON THE FERMENTATION MEDIUM CONTAINING MOLASSES, YEAST FODDER AND RICE BRAN.

Conc.	Final pH	Suspended dry matter (mg/ml)	Oxytetracycline (μg/ml)
(Different con. of molasses (g/l)			
10	7.5	6.5	350
15	8.0	7.0	400
20	8.0	7.0	450
25	8.5	7.5	500
30	8.5	8.0	550
40	8.0	8.5	500
50	8.0	8.5	300
60	7.0	8.5	200
70	7.0	8.5	150
Molasses (30 g/l) + different conc. of fodder yeast (g/l).			
2.5	8.0	6.5	500
5.0	8.0	7.0	550
10.0	8.0	7.5	600
15.0	8.0	7.5	750
20.0	8.5	8.5	800
25.0	8.0	9.5	700
30.0	7.5	10.0	650
Molasses (30 g/l) + different conc. of rice bran			
10.0	7.5	7.0	450
20.0	7.5	8.0	450
30	8.5	9.5	600
40	8.5	10.0	600
50	8.0	10.5	500
Molasses (30.0 g/l) + fodder yeast (20.0 g/l + different conc. of rice bran			
5	7.5	9.0	650
10	7.5	10.0	850
15	8.0	10.5	750
20	8.5	11.0	700
25	8.5	11.5	600
30	8.0	12.0	600
40	7.0	12.0	600
Molasses (30.0 g/l) + rice bran (10.0 g/l + different conc. of fodder yeast			
5	7.0	10.0	800
10	7.0	10.5	850
15	8.0	11.0	950
20	8.0	12.0	1000
25	8.0	12.5	950
30	7.5	12.5	800
35	7.5	13.5	750
40	7.5	14.5	750
45	7.0	15.0	700
50	7.0	15.0	700
Molasses (30.0 g/l) + fodder yeast 20.0 g/l + rice bran 10.0 g/l) + different conc. of KH_2PO_4			
0.00	7.5	9.5	1000
0.10	7.5	9.5	1200
0.20	7.5	10.5	1250
0.30	8.5	11.5	1000
0.40	8.5	12.5	1000
0.50	8.5	12.5	950
0.75	8.0	11.5	800
1.00	8.0	12.0	800
1.50	8.0	12.0	750
2.00	8.0	12.0	700
Molasses (30.0 g/l) + fodder yeast (20.0 g/l + rice bran (10.0 g/l) KH_2PO_4 0.2 + different conc. of $MgSO_4 . 7H_2O$			
0.00	7.0	8.5	1000
0.10	7.0	8.5	950
0.20	7.0	9.5	950
0.30	7.5	10.5	900
0.40	8.0	11.0	800
0.50	8.5	12.0	750
0.75	8.0	11.0	700
1.00	8.0	11.0	700

tetracycline was biologically assayed to represent 750 μg/mg of the crude oxytetracycline.

Discussion

Streptomyces rimosus 12907 of Institute for Fermentation of Japan gave a high antibiotic yield, more than *Streptomycesrimosus* Finlay *et al.*, 93060 and *Streptomyces rimosus* NRRL 2234. The composition of the fermentation medium plays an important role in the antibiotic production as well as the strain of the antibiotic producer. Medium I was suitable as the fermentation medium for the production of oxytetracycline. But this medium contained ingredients which were expensive for the production of oxytetracycline on a large scale, therefore, it was preferable to replace these components by other available cheap raw materials. Black strap molasses (30.0 g/l) replaced glucose; fodder yeast (40% total protein) (20.0 g/l besides undefatted rice bran (10.0 g/l) replaced soybean meal, while the other ingredients remained constant. The addition of KH_2PO_4 (0.2 g/l) increased efficiency of the fermentation medium for high antibiotic yield, while $MgSO_4$ did not promote it.

Fodder yeast (40% total protein) is produced locally from Egyptian black strap molasses using *Saccharomyces cerevisiae.* Fodder yeast contains 40% total protein, ash (9.0 - 10.0%), water (5.0%), vitamins, ergosterol and other organic ingredients. It is cheaper than soybean meal.

High levels of antibiotic titre were produced when the microorganism was cultured on medium containing molasses, fodder yeast (40% total proteins) and rice bran, and this may be ascribed to the presence of micronutrients as well as other growth factors and vitamins which always occur in reasonable quantities in the nonsynthetic medium. Another reason for the improved yields obtained with nonsynthetic medium is due to the presence of readily available organic compounds which the microorganism would have to synthesise from simple structural units.

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