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EFFECT OF SODIUM CHLORIDE AND FLUORIDE ON THE SELECTIVE PRODUCTION OF THE TETRACYCLINE ANTIBIOTICS BY STREPTOMYCES AUREOFACIENS

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The tetracycline antibiotics are closely related antimicrobial metabolites. The constituents of the fermentation medium are very important for the selective production of chlortetracycline or tetracycline. The basal fermentation medium was able to produce about 64.3% CTC and 35.7% TC. On increasing the concentration of NaCl, the production of CTC increased. When the NaCl level was 80 mg/l in the basal medium, the percentage of CTC and CT was about 90.6 and 9.4% respectively. On the contrary, increasing the concentration of NaF in the fermentation medium increased the production of TC. Therefore, at 80 mg NaF/l the percentage of TC and CTC was 99.4 and 0.6 respectively. To nullify the effect of the chloride level present in the fermentation medium, different concentrations of NaF must be added. The basal medium and 80 mg NaF/l were able to produce TC (100%).

The tetracycline-producing strains are not specific since they can also produce chlortetracycline. *Streptomyces aureofaciens*, the usual chlortetracycline producer, may almost wholly, produce tetracycline under appropriate conditions. Rolland and Sensi[1] demonstrated that the ratio of the production of one rather than the other antibiotic depends on chlorine concentration. This metabolic process of active chlorination by *Streptomyces aureofaciens* may be antagonized by the presence in the fermentation medium of suitable inhibitors such as bromide, iodine, or thiocyanate.[2,3,4]

The aim of this paper is to investigate the effect of sodium chloride and fluoride concentrations on the selective production of the tetracycline antibiotics by *Streptomyces aureofaciens*.

Materials

Maintenance of Streptomyces aureofaciens.—The microorganism was 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 and distilled water up to 1000 ml. The initial pH of the medium was adjusted to 7.0 before sterilization. The slants were inoculated with *Streptomyces aureofaciens* under aseptic conditions and they were incubated at 27–30°C for 10 days to permit good sporulation. Then, the slants were kept in a refrigerator at 5°C.

Preparation of the Vegetative Inoculum.—The microorganism was cultured on the medium containing these nutrients (g/l): corn steep liquor 20.0 and starch 15.0. The initial pH of the medium was adjusted to 7.0 before sterilization. 250 ml Erlenmeyer flasks, each containing 50 ml of the medium were sterilized at 21.5 lb/in^2 for 20 min. The flasks were inoculated with *Streptomyces aureofaciens* under aseptic conditions. They were inserted on a rotory shaker (200 rev/min) at 27–30°C for 72 hr to permit good growth of the microorganism to be used as the spore suspension ready for inoculating the medium used for the production of tetracyclines.

Production of the Tetracycline Antibiotics.—The medium used for the production of the tetracycline antibiotics contained the following ingredients (g/l): corn steep liquor 5.0, NH_4NO_3 5.0, $CaCO_3$ 5.0, NaCl 2.0, starch 30.0. The initial pH of the medium was adjusted to 7.0 before sterilization. Erlenmeyer flasks (250 ml capacity) each containing 50 ml of the medium were sterilized at 21.5 lb/in^2 for 20 min. The flasks were inoculated with a standard inoculum of the spore suspension of *Streptomyces aureofaciens* under aseptic conditions. The inoculated flasks were inserted on a rotary shaker (200 rev/min) at 27–30°C for 72 hr.

At the end of the incubation period, the total tetracyclines (chlortetracycline and tetracycline) were determined[5], whereas, chlortetracycline and tetracycline were estimated bioautographically[6] using *Bacillus subtilis* NRRL B-543 as a sensitive microorganism. Buffered strips (pH 3.0 phosphate buffer) of Whatman No. 1 (1 × 45 cm) were spotted by known volumes of the chloroform extract of the fermentation broth. The strips were developed descendingly using n-butanol saturated with water. On completion, the developed strips were dried and exposed to ammonia for 15 min to make them neutral. The strips were placed on the surface of the seeded medium containing the following ingredients (g/l): glucose 10.0, peptone 6.0, yeast extract 3.0, meat extract 1.5, agar agar 20.0 and distilled water upto 1000 ml using *Bacillus subtilis* NRRL B-543 as the test organism. The trays containing the seeded medium and the developed chromatographic strips were incubated in a refrigerator at 5°C for 2 hr to permit good diffusion. Then, the trays were incubated at 37°C for 24 hr to measure the inhibitory zones of chlortetracycline and tetracycline exhibited by the separation of the two antibiotics. The amounts of both antibiotics were calculated from the drawn standard biological curves.

Results

Effect of the Increasing Concentration of Chloride Level in the Basal Medium on the Production of the Tetracycline Antibiotics.—The influence of the different concentrations of chloride level supplemented into the basal medium is represented in Table 1. The data showed that the basal medium (without NaCl) supported the microorganism for the production of tetracycline and chlortetracycline. The production of chlortetracycline in the basal medium was due to the presence of some chloride ions as impurities in the ingredients. The percentage of CTC to the total antibiotics (tetracycline and chlortetracycline) amounted to about 64.3 in the basal medium. On increasing the concentration of the chloride level, the production of CTC was increased. At high concentration of the chloride level (80 ml/l), the percentage of CTC was about 90.6. On the contrary, the production of tetracycline (TC) decreased with the increase of the concentration of the chloride level in the basal medium reached its minimal percentage 9.4 (at 80 mg/l). At high NaCl concentration (80 mg/l), a decrease in the production of both antibiotics was recorded. The initial pH of the fermentation medium was 7.0 and, after the fermentation, the final pH shifted towards alkaline side (8.22–8.40). The mycelial dry weight was in the range of 5.0–5.6 mg/ml.

Effect of the Increasing Concentration of Fluoride Level in the Basal Medium on the Production of the Tetracycline Antibiotics.—The effect of increasing the concentration of fluoride level in the basal medium on the production of the tetracycline antibiotics is reported in Table 2. The results indicated that basal medium favoured the production of the two antibiotics and the percentage of TC to the total antibiotics (TC and CTC) was about 34.6. On increasing the concentration of the fluoride level, the production of TC increased reaching its maximal percentage of about 99.4 at 80 mg/l NaF. On the contrary, the increase of NaF concentration in the basal medium led to the reduction of the production of CTC, therefore, at low concentrations of NaF, the productions of CTC was high and it was minimum at high concentrations of NaF. At 80 mg/l NaF, the percentage of the CTC production was about 0.6. The initial pH of the basal medium was 7.0 and at the end of the incubation period it was towards the alkaline side (8.25–8.35). The mycelial growth of the microorganism was in the range of 5.50–5.67 mg/ml.

Effect of the Different Concentrations of NaCl and NaF on the Selective Production of CTC and TC.—The influence of different concentrations of NaCl and NaF on the production of CTC and TC is recorded in Table 3. The results showed that without NaCl and 80 mg/l NaF, the end product was mainly tetracycline, while at 80 mg/l NaCl and without NaF, the end product was mainly

Table 1.—Producibilities of Mycelia for Chlortetracycline (CTC) and Tetracycline (TC) when *St. aureofaciens* Grown on the Basal Medium Supplemented with Different Concentrations of NaCl.

NaCl (mg/l)	Final pH*	Mycelial dry wt mg/ml	Total tetracyclines µg/ml	TC (µg/ml)	CTC µg/ml
00	8.30	5.60	965	344	621
01	8.30	5.60	965	333	632
05	8.30	5.60	961	196	765
10	8.25	5.50	965	153	812
20	8.30	5.60	962	106	856
40	8.22	5.55	936	048	888
60	8.40	5.00	958	102	856
80	8.30	5.00	901	073	828

*Initial pH of the basal medium was 7.0

Table 2.—Producibilities of Mycelia for the Production of Chlortetracycline (CTC) and Tetracycline (TC) when *St. aureofaciens* Grown on the Basal Medium Supplemented with Different Concentrations of NaF.

NaF (mg/l)	Final pH*	Mycelial dry wt (mg/ml)	Total tetracycline (µg/ml)	TC (µg/ml)	CTC (µg/ml)
0	8.30	5.60	968	335	633
1	8.30	5.63	966	430	536
5	8.35	5.59	971	725	246
10	8.25	5.55	962	738	224
20	8.30	5.67	972	764	208
40	8.33	5.60	911	896	015
60	8.33	5.50	908	899	009
80	8.35	5.50	871	866	005

*Initial pH of the basal medium was 7.0.

Table 3.—Producibilities of Mycelia for the Production of Chlortetracycline (CTC) and Tetracycline (TC) when *St. aureofaciens* Grown on the Basal Medium Supplemented with Different Concentrations of NaCl and NaF.

NaCl (mg/l)	NaF (mg/l)	Final pH*	Mycelial dry wt. mg/ml	Total tetracycline ug/ml	TC (ug/ml)	CTC (ug/ml)
00	80	8.20	5.90	890	890	—
01	60	8.30	5.90	905	884	21
05	40	8.25	6.10	912	890	22
01	20	8.30	6.30	930	735	195
20	10	8.35	6.20	950	661	289
40	05	8.33	5.30	960	378	582
60	01	8.30	5.00	956	194	762
80	00	8.30	5.00	900	049	851

*Initial pH of the basal medium was 7.0.

chlortetracycline. On increasing the concentration of NaCl and decreasing the concentration of NaF, the production of CTC increased and the biosynthesis of TC increased by decreasing the concentration of NaCl and increasing the concentration of NaF. The percentage of CTC and TC at (0 mg/l NaCl and 80 mg/l NaF) was 0 and 100 respectively, while at (80 mg/l NaCl and 0 mg/l NaF) the percentage of CTC and TC was 94.5 and 5.5. The initial pH of the basal medium was 7.0 and at the end of the incubation period, the final pH was towards the alkaline side (8.20–8.35) and the mycelial growth was in the range of 5.0–6.3 mg/ml.

Discussion

The constituents of the fermentation medium are of important factors for the production of the tetracycline antibiotics by *St. aureofaciens*. Therefore, when TC is required to be the main end product, the basal medium must be dechlorinated, thus forcing the microorganism to produce only tetracycline or the presence of the chloride ions may be antagonized by the presence in the fermentation media of suitable inhibitors such as fluoride. On increasing the chloride level the chlortetracycline percentage rises while the tetracycline percentage decreases and, on the contrary, on increasing the fluoride level the tetracycline percentage rises while the chlortetracycline percentage decreases.

A variety of compounds both cyclic and acyclic have been found to possess activity as chlorination inhibitors for *Streptomyces aureofaciens* in that they block the normal chlortetracycline pathway in favour of the tetracycline pathway of biosynthesis.[7] When the fermentation medium was supplemented with a halide ion such as NaBr, KBr, $CaBr_2$, NH_4Br, HBr or NaCN, fermentation was directed to the formation of tetracycline.[8,9,10,11] NaF, NaBr, or NaI were found to be as antimetabolites of chloride ions in that the bromide seemed to inhibit the production of chlortetracycline by competing with chloride ion in the chlorination reaction in the biosynthesis of chlortetracycline but NaF was thought to inhibit the biosynthetic system itself by inhibiting dephosphorylation of high energy phosphate essential for the biosynthesis.[12]

It is improbable that chlortetracycline is produced directly from tetracycline through simple chlorination reaction. The same precursor of tetracycline is chlorinated at the same point in the chain of biosynthetic reactions. Then after the precursor is chlorinated, it undergoes further synthetic reactions which culminate in CTC. Tetracycline is produced from non-halogenated precursors.

It may be deduced that tetracycline may be obtained by the following fermentation processes: (a) addition of inhibitors of chloride utilization to the usual media composed of organic ingredients, (b) fermentation on synthetic media free of chloride, (c) elimination of chloride by chemical means from the usual media containing organic ingredients. Forbath[7] reported that tetracycline may be produced by dechlorination and catalytical hydrogenation of the fermentation media. Sikyta and Herold[8] discussed the replacement of the usual sources of organic nitrogen in the nutrient medium with peanut flour (ground *Arachis hypogaea*) caused a drop in the tetracycline production to lower than 10% of the total amount of the antibiotics and without the peanut flour the amount of tetracycline was 20.0% of the total antibiotics produced.

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