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Studies on the Production of Chlortetracycline

(Part 1) The Effect of Carbon Source and Mineral Ions

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Chlortetracycline 醱酵에 關한 硏究

(第 1 報) 炭素源과 2 價金屬이온의 影響

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Abstract

Improvement of fermentation medium for chlortetracycline production was attempted. Starch was a good carbon source for chlortetracycline production as compared with sucrose or glucose, although the cell yield from the starch medium was somewhat lower, and complexed natural medium was more suitable than artificial and simple one for this purpose. The concentrations of divalent ions, such as Mg^{2+}, Mn^{2+}, Zn^{2+} and Fe^{2+}, affected the productivity of chlortetracycline. These mineral ions in excess of which was contained in natural medium such as corn flour and corn steep liquor, caused significant decrease in the productivity of chlortetracycline.

Introduction

Many workers have studied on the medium improvement for chlortetracycline production, Zelinka, et al.[1] studied 32 organic nitrogen sources for their effects on the biosynthesis of chlortetracycline. McCormic, et al.[2] tested 31 organic nitrogen sources and found only two, L-histidine and L-methionine, that gave reproducible stimulation of chlortetracycline production. Comparative tests were also made by van Dyck et al.[3] using several organic nitrogen compounds to determine their effects on chlortetracycline production. Backus et al.[4] studied the effect of carbohydrate source on productivity of tetracycline. B.M. Duggar et al.[5] suggested such mineral salts as magnesium, zinc, manganese, and iron that were considered required for production of tetracycline. Nidercorn[6] also stressed the importance of calcium and magnesium cations in chlortetracycline fermentation media.

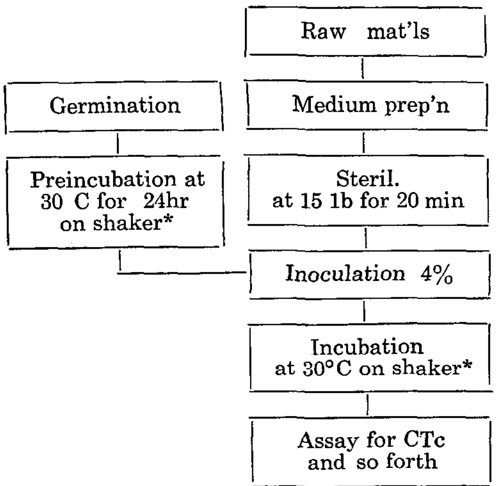
Van Dyck and P. De Somme[3] studied the effect of different saccharides on antibiotic production. In this study, sucrose was found to be the best among several carbohydrates for chlortetracycline production. Van Dyck, et al.[3] reported that iron ion has a detrimental influence on chlortetracycline production, which was contradictory to the results fo Duggar.[5]

It is generally known that acetyl-Co A is the precursor in the biosynthesis of tetracycline derivatives, but when acetic acid or sodium acetate was added to the fermentation medium they did not affect on chlortetracycline production.[4]

In this experiment, medium improvement was attempted because chlortetracycline yield wss relatively low when conventional medium reported in the literature was used. We found that there are many factors related to the medium that affect the production of chlortetracycline. Among them, the concentration of divalent ions and the kind of carbohydrate sources were believed to be rather important. Thus, we studied the effect of divalent ions and carbohydrate sources on chlortetracycline production using *Streptomyces aureofaciens (ATCC 13900).*

Experiment

Control fermentations were performed as follows: A spore suspension of *Streptomyces aureofaciens* (ATCC 13900), was inoculated into a slant which was made of Emerson agar medium. After incubation for 2 to 5 days at 30°C, this was transferred into 100ml medium: of the following: Corn flour, 3%; corn steep liquor, 4%; CaCO , 0.9%; corn starch, 5%; ammonium sulfate, 0.7%; ammonium chloride, 0.1%; cobalt chloride, 0.5 mg %; rice bran oil, 2%. The fermentations were carried out in 500 ml shaking flasks at 30 C on a reciprocating shaker (3.6 cm stroke at 140 cycles per min.). The flow sheet for chlortetracycline (CTc) fermentation is shown in Fig. 1. After 24 hrs, 4 ml culture fluid was transferred into 96 ml of the fermentation medium which has the same composition as described above. Media were sterilized at 120 C for 20 minutes.



* Reciprocating with 3.6 cm stroke at 140 cycles per min.

Fig. 1. Fermentation Procedure for Chlortetracycline.

Aliquots were taken from the fermentation flasks at suitable intervals of time and the following assays were performed:

1. Dry weight

5 or 10 ml broth samples were acidified with HCl in order to dissolve the calcium salts, then filtered. After two washings, the filter cake was dried and weighed.

2. Total hyphal length

Aliquot of fermented sample were blended till reasonably homogeneous fragmented hyphae were obtained and diluted to appropriate level. Total hyphal length were determined with the aid of ocular micrometer, hemacytometer and microscope. This result was used for comparison of the total hyphal length with the dry cell weight.

3. Chlortetracycline was assayed by spectrophotometrically

Aliquot of fermented broth were sampled. After filtration with the aid of filter paper, the filter cakes were washed with acidified water (pH 3.5 to 3.7). The pH of the filtrate was adjusted to 3.7 with HCl and diluted to a level adequate for accurate assay. Chlortetracycline was extracted with butanol[1] and the absorbbance at 365 nm was read. The flow sheet of this procedure is shown in Fig. 2.

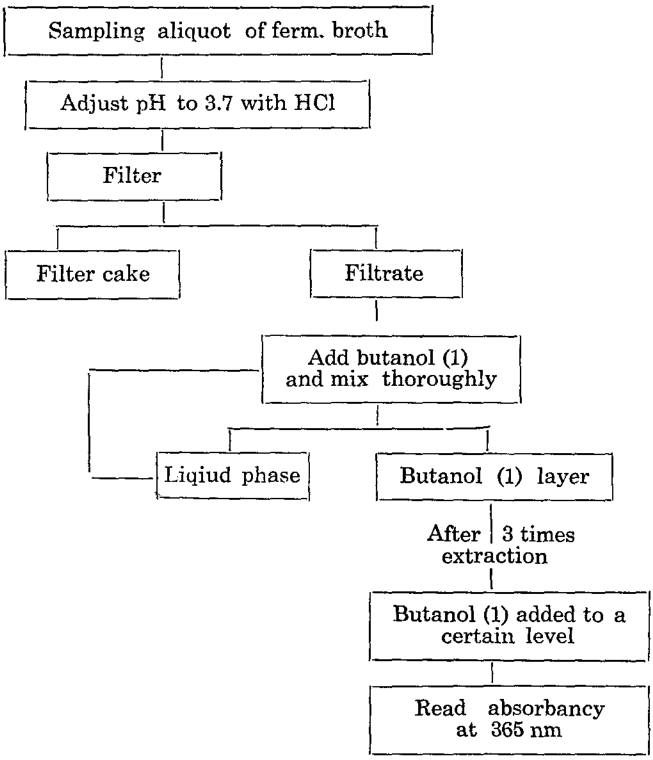


Fig. 2. Assay Procedure for Chlortetracycline.

4. Mineral ions

The concentrations of mineral ions were assayed by atomic absorption spectrophotometer.

5. Identification of chlortetracycline

The identification of chlortetracycline was performed using thin layer chromatography. The developing solvent is made of a mixture of butanol, methanol and 10% citrate (3:4:3). The Rf values of chlortetracycline standard obtained from NIH and the sample from fermentation broth were both about 0.44.

Result and Discussion

In the preliminary experiment, chlortetracycline production was very sensitive to the medium composition, especially for carbohydrate sources and mineral ions[1~4]. To study this, we established the basal medium composition as

$CaCO$, 9 g: $(NH_4)_2 SO_2$, 7 g; NH_4Cl, 1 g; $CoCl_2 \cdot 6H\ O$, 0.005 g; rice bran oil, 20 ml 'per liter of medium.

1. Effect of different carbon sources on chlortetracycline production

We studied the effects of medium compositions on chlortetracycline production and as shown in Table 1, we could conclude that complexed natural medium component was more suitable than chemically defined simple medium for chlortetracycline production and carbohydrate sources also showed much drastic effect on chlortetracycline production.

Table 1. Effects of Medium Composition on Chlortetracycline Production.

	Medium Composition (per liter)*				
	Glucose (40 g)	Glucose (40 g)+ Sucrose (20 g)	Sucrose (20 g)	Sucrose(30 g) corn starch (30 g)	Corn stach (50 g)
Yeast (1g)	—	—	0.32	0.52	0.78
Corn steep liquor (50 ml)	—	—	0.56	0.68	0.95
C.S.L. (50 ml) + corn flour (30 g)	—	0.34	0.58	0.74	1.00

* The above medium composition was added to the basal medium composed of $CaCO$, 9 g; $(NH_4)_2SO_4$, 7 g; NH_4Cl, 1 g; $CoCl_2 \cdot 6H_2\ O$, 0.005 g; rice bran oil, 20 ml in 1 liter.

* Relative productivity: 1.00

When glucose unit was increased, e.g., glucose to starch, chlortetracycline production was also increased. Of course, partial data for this result was shown in previous works[7~9], but in some cases they used only sucrose for carbohydrate source and in other case, they used starch for this purpose. In this study, we tried to establish the comparable data for carbohydrates for the production of chlortetracycline. For this purpose, we studied in detail for the effect carbohydrates. As shown in Table 2, starch was the best one for chlortetracycline production, but glucose or sucrose is more suitable than starch for cell growth.

From the above data, we could deduce the following inferences:

1) When we add glucose or sucrose to the fermentation medium, the microbes can easily utilize them for cellular metabolism, thus there is negligible need for the production of new enzymes and now metabolic path way which will produce amino acids, peptides, etc. In this case, only cell growth will occur and whit the environment become bad, then they will produce antibiotics as by-product.
2) When starch are abundant in the fermentation medium, the microbes should produce certain kind of enzymes to utilize starch for their metabolism. During the course of enzyme production, amino acids, peptides or other metabolites which is essential for antibiotics production will be produced. In this case, cell growth was inhibited to a certain level and chlortetracycline production was activated very highly.

Table 2. Effects of Different Carbon Sources on Chlortetracycline Production and Cell Growth.

Carbon source at 5 percent	Relative productivity of chlortetracycline	Relative dry weight
Sucrose	0.55	1.81
Glucose	0.11	1.72
Starch	1.00	1.00

From the above data, we concluded that the medium composition shown in Table 3, was optimal for chlortetracycline when the mineral ions were not considered.

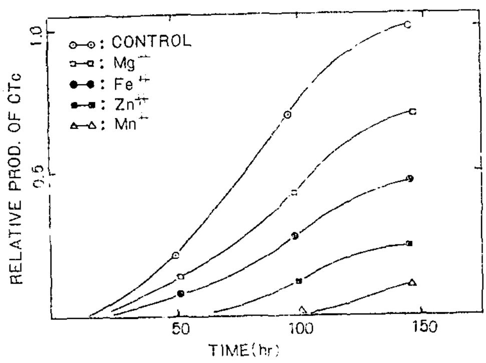
Table 3. Medium Composition* (per liter).

Corn flour	30 g
Corn starch	50 g
$CaCO_3$	9 g
$(NH_4)_2SO_4$	7 g
NH_4Cl	1 g
$CoCl_2 \cdot 6H_2O$	0.005 g
Corn steep liq.	40 ml
Rice bran oil	20 ml
pH adjusted to 6.8~7.0 with HCl	

* This medium was used as control throughout the whole experiment.

2. Effects of divalent ions on chortetracycline production

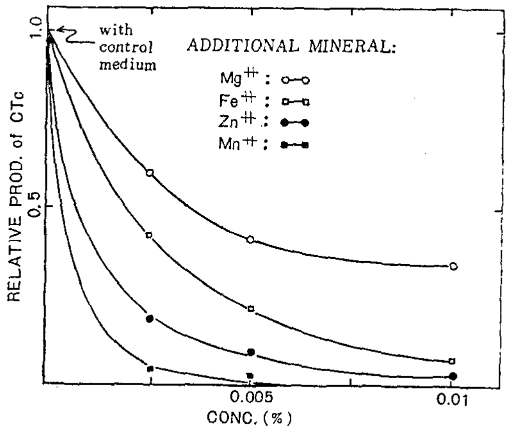
Fig. 3 shows the productivity profile of chlortetracycline as the effect of divalent ions during fermentation. From this figure, we could find that the total amount of chlortetracycline produced during fermentation was significantly affected by different kind of mineral ions. We also found that the productivity of chlortetracycline of control experiment was the best one. The medium for the control experiment contained only those mineral ions that was carried in with the natural raw material such as corn steep liquor and corn flour except cobalt chloride.



* The concentration of mineral ions is [illegible]25%.

Fig. 3. Effects of Divalent Ions with Constant Concentration on Chlortetracycline Productivity.

Fig. 4 shows the effect of the concentration of divalent ions on the productivity of chlortetracycline. The data show that even the addition of very small amount of divalent ions causes decrease in chlortetracycline productivity for the given organism used in this study. In case of manganese, zinc and ferrous iron, with the increase of their concentrations the production of chlortetracycline decreased sharply.



* Sampling after incubation for 144 hrs.

Fig. 4. Chlortetracycline Production for Several Concentration of Minerals.

To verify the concentration of divalent ions which could be optimal, we checked the concentration of divalent ions included in the natural medium as shown in Table 4. Even though we could not determine the optimal concentration of divalent ions exactly, we studied the effect of the trace variation of divalent ions with the concentration change of natural medium and thus we could not check only the effect of divalent ions, we concluded that the concentration of divalent ions included in the natural medium were enough for chlortetracycline production and when we added them more to the medium, we only could obtain lowered chlortetracycline product. Here we also concluded that medium composition shown in Table 3 was the most improved one for this experimental condition.

Table 4. Concentration of Minerals in Corn Flour and Corn Steep Liquor.

	Corn flour	Corn steep liq.
Mg^{2+}	1,000 ppm	1,065 ppm
Mn^{2+}	0 ppm	5.2 ppm
Zn^{2+}	30 ppm	12.5 ppm
Fe^{2+}	16.7 ppm	8.9 ppm

Conclusion

In conclusion we compared carbohydrate sources for medium improvement for chlortetracycline production and concluded starch was the best one for this purpose.

We concluded that complexed natural medium was more suitable than artificial and simple one.

Divalent ions involved in natural medium was enough and the addition of very small amount of them to the medium showed negative effect only.

We obtained the most improved medium composition for chlortetracycline production as shown in Table 3 for this experimental condition.

요 약

지금까지의 여러 연구 보고에서 나타난 배지 성분 중 공통적인 것을 간추려 발효를 진행한 결과 미생물 성장과 항생제 발효가 아주 좋지 않았다. 여러가지로 배지 조성을 바꾸어 본 결과 2가 금속들과 탄수화물이 아주 중요한 요인임을 알 수있었다. 2가 금속들은 천연배지에 들어있는 이상으로 첨가될 때는 일반적으로 항생제 생산을 배제하는 영향을 보였다. 탄수화물은 지금 까지의 연구결과와는 달리 전분이 설탕보다 항생제 생산에 효과가 큼을 알 수 있었다. 또한 배지성분이 화학적으로 성분이 알려진 단순한 경우보다 천연 배지로서 여러가지 복합된 경우가 더욱 효과적임을 확인하였다.

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