STUDIES IN BACTERIAL AMYLASE

III. INFLUENCE OF THE CONCENTRATION OF THE CULTURAL NUTRIENTS ON THE FORMATION OF BACTERIAL AMYLASE

by

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In previous communications[1,2], methods have been described for the cultivation of a highly amylolytic strain of *B. subtilis* on a synthetic medium containing a source of nitrogen, a source of carbon and mineral salts. Amongst the different sources of carbon studied, starch has been found to be highly conductive to the maximum secretion of the enzyme. Of the sources of nitrogen, ammonium lactate has given the best yield. In these experiments the ratio between nitrogen and carbon has been maintained at 1:13. These studies, however, led to speculation whether variation in this ratio could possibly influence the amylase yield, especially in view of the work of WALDMANN[3], who in his patent on amylase production has recommended the employment of one part of nitrogen to at least 40 parts of carbohydrate to secure a maximum yield of bacterial amylase. The object of the present investigation has therefore been to investigate: a. the optimum ratio of nitrogen to carbohydrate for obtaining the maximum yield of amylase from *B. subtilis* (N.C.T.C., 2027N) and b. the influence of the cultural inorganic salts in enhancing the amylase formation.

a. *Optimum ratio of nitrogen to carbohydrate*

These experiments have been conducted employing starch as the source of carbohydrate and ammonium nitrate as the only source of nitrogen instead of ammonium lactate which was previously employed[1]. This was necessary as the lactate radical furnished additional carbon to the culture medium. The composition of the culture medium consisted of a 2.0 mg level of ammonium nitrate, varying amounts of starch to obtain different ratios between nitrogen and carbohydrate, 1.0 ml of salt solution, p_H adjusted to 7.0 and finally the total volume made up to 10 ml in each case. Except for these deviations, the cultural conditions and the method of determining the amylase activity were same as described earlier[1]. The results are expressed in enzyme units and are given in Table I and Fig. 1.

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TABLE I

INFLUENCE OF THE AMMONIUM NITRATE TO CARBOHYDRATE RATIO ON THE FORMATION OF AMYLASE

Carbohydrate level (mg)	Ratio in the medium	Growth observation (hours)			Amylase units per 10 ml of the medium
		24	48	72	
20	1:10	—	+	+	0.0
40	1:20	+	++	++	23.0
60	1:30	++	+++	+++	45.4
80	1:40	+++	++++	++++	52.6
100	1:50	++++	+++++	+++++	58.0
120	1:60	+++++	++++++	++++++	52.2

It is evident from the results presented in Table I and Fig. 1 that the concentration of the starch in the culture medium greatly influences the formation of amylase. Observation of the growth of the bacterium indicated that the organism multiplies with much ease as the concentration of the carbohydrate is increased in the culture medium. The H-ion concentration of the culture medium after the growth was found to be in the neighbourhood of 7.0 and so there was no appreciable variation in the initial and final p_H of the medium. The maximum amylase formation took place when 100 mg of starch were present in the culture medium. At higher concentrations of starch (120 mg), the amylase yield was found to decrease. Fig. 1 gives such values obtained with different concentrations employed. An interesting observation has been made during these investigations that the bacterial growth is quite independent of amylase yields at starch concentrations above the optimum for amylase formation. The extent of bacterial growth and amylase formation increase as the starch concentration is increased up to a level of 100 mg, which gives a ratio of 1:50 of nitrogen to carbohydrate. Beyond this ratio, however, amylase secretion diminishes continuously while the growth still increases with the increase in carbohydrate concentration.

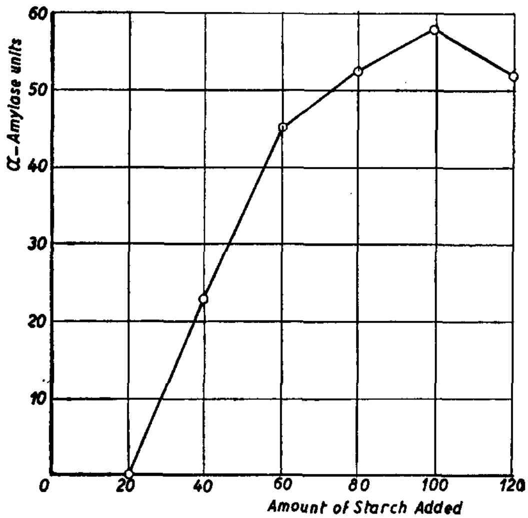

Fig. 1. Influence of the nitrogen to carbohydrate ratio on the amylase formation

The above results clearly indicate that the ratio 1:50 of ammonium nitrate to carbohydrate offers the most favourable concentration of nutrients for the organism to produce a higher proportion of amylase. These findings suggested an investigation as to whether higher amounts of ammonium nitrate and carbohydrate, while maintaining the ratio 1:50, favour better yields of the enzyme. The results of these investigations are given in Table II and Figs. 2 and 3.

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TABLE II

INFLUENCE OF INCREASING AMOUNTS OF AMMONIUM NITRATE AND STARCH AT THE RATIO 1:50 ON THE FORMATION OF AMYLASE

mg NH_4NO_3 per 10 ml	mg starch per 10 ml	p_H after growth	Amylase units per 10 ml of the medium
2	100	7.1–7.2	11.2
4	200	7.1–7.2	50.0
6	300	7.1–7.2	96.1
8	400	7.1–7.2	105.8
10	500	5.3–5.5	47.3
12	600	5.3–5.5	35.3

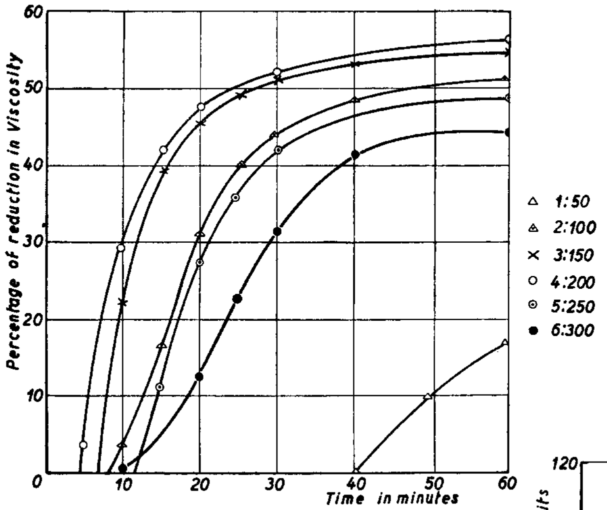

Fig. 2. Influence of the multiples of the optimum ratio (N:C) on the formation of amylase

These clearly indicate that the optimal amylase formation is not only determined by the ratio of NH_4 nitrate to carbohydrate, but also by the amounts of these substances. The maximum concentration of amylase is obtained when 8 mg of ammonium nitrate and 400 mg of starch are present per 10 ml of bacterial suspension. Higher amounts, while maintaining the ratio 1:50, do not yield an appreciable concentration of the enzyme and this has been found to be due to a change in the p_H of the culture medium during the bacterial growth. The p_H in these culture tubes was shown to be 5.5 after 72 hours' growth. This indicates that the lower enzymic yield at higher concentrations of ammonium nitrate and starch is probably due to the destruction of the amylase at this p_H. On the other hand, the bacterial growth was much better at higher multiples than at lower multiples.

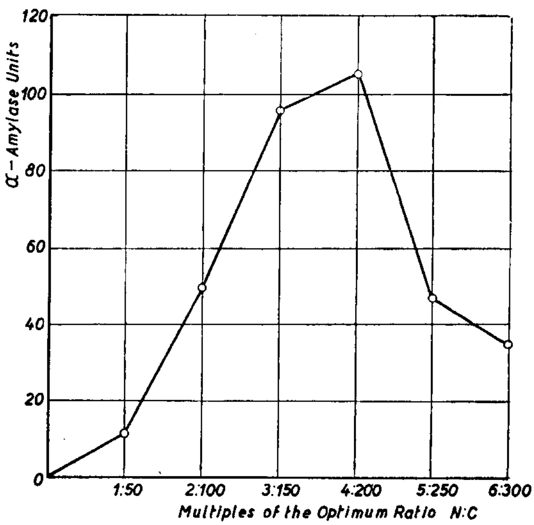

Fig. 3. Effect of the multiples of optimum ration (1:50) on the production of bacterial amylase

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b. *Effect of mineral salts in the formation of amylase*

Inorganic salts have played a very important role in the growth and metabolic function of micro-organisms. The optimum concentration of the essential salts and the tracer elements have yielded many a time a vigorous fermentation and a concomitant greater fermentation efficiency in the resulting product. The present studies have been directed to find out the effect of individual inorganic salts employed in the culture medium throughout the earlier studies on the formation of bacterial amylase. The technique was carried out for this purpose as follows: a control containing all the inorganic ingredients of the Basal Medium was compared with an experimental series, each lacking in one of the inorganic salts under investigation. The composition of Basal Medium or the inorganic solution was as follows:

$K_2HPO_4 \cdot 3H_2O$	1.0 g
NaCl	0.5 g
$CaCl_2$	0.1 g
$MgSO_4 \cdot 7H_2O$	0.1 g
$MnSO_4 \cdot 7H_2O$	0.01 g
$FeSO_4$	0.01 g

Total volume made up to 100 ml

Except for these deviations, the methods employed for growing the culture of *B. subtilis;* N.C.T.C., 2027 N and finally for assaying the amylase activity were the same as described above. The results are given in Table III and Fig. 4.

TABLE III

EFFECT OF DIFFERENT INORGANIC SALTS ON THE FORMATION OF AMYLASE

Inorganic salts	Amylase units per 10 ml of the medium
Complete Basal Medium	40.0
B.M., K_2HPO_4 omitted	0.0
B.M., NaCl omitted	19.1
B.M., $CaCl_2$ omitted	18.0
B.M., $MgSO_4$ omitted	27.4
B.M., $MnSO_4$ omitted	33.5
B.M., $FeSO_4$ omitted	22.5

From Table III and Fig. 4, it is evident that the salts of Basal Medium are indispensable for the formation of amylase. The absence of one of these members of the group results in a low amylase formation. KH_2PO_4 is found to be very essential for the growth and amylase secretion as the bacteria refuse to grow on the media lacking in this particular ingredient. The salts of Basal Medium exert their beneficial influence on the amylase formation in the following order:

$$K_2HPO_4 > CaCl_2 > NaCl > FeSO_4 > MgSO_4 > MnSO_4$$

WALLERSTEIN[4] has reported in general that for the production of microbial enzyme essentially calcium and magnesium, etc., are required. BECKORD *et al.*[5] have made use of mineral salts such as K_2HPO_4, $MnSO_4 \cdot 7H_2O$, NaCl, and trace elements like H_3BO_3,

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$FeSO_4$, $MnSO_4 \cdot 7H_2O$, $CaCl_2$, $ZnCl_2$, for the production of bacterial amylase by *B. subtilis*. In order to study the effect of the different concentration of these essential salts, the

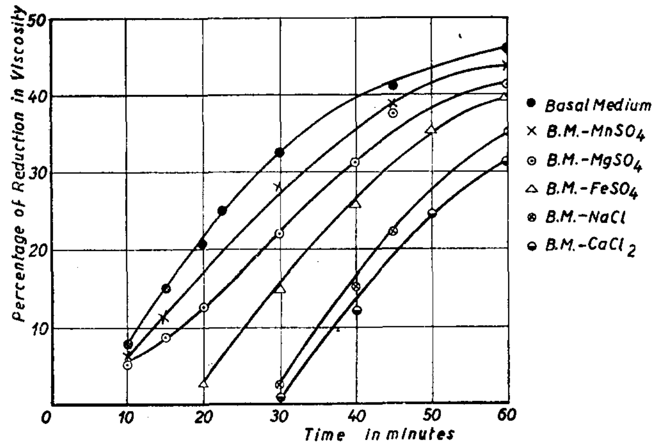

Fig. 4. Effect of different inorganic salts on the formation of amylase

same technique of compounding the media was employed. In each experimental series the effect of different concentrations of the salt was tested. The results obtained at different concentrations of the various salts have been presented in Table IV and Figs. 5 and 6.

TABLE IV

INFLUENCE OF THE CONCENTRATION OF THE SALTS ON THE AMYLASE FORMATION

mg salt per 10 ml medium	Amylase units per 10 ml of the medium						
	K_2HPO_4	$CaCl_2$	NaCl	$MgSO_4$	$MnSO_4$	$FeSO_4$	$CuSO_4$
0.0	—	18.2	25.0	30.0	33.9	20.5	37.5
0.1	—	—	—	—	39.0	48.5	36.0
0.2	—	—	23.8	37.8	43.1	—	37.0
0.5	—	24.3	—	40.0	42.0	40.0	36.5
1.0	0.0	32.0	29.2	38.0	42.5	35.5	32.0
2.0	0.0	43.0	32.0	36.2	37.5	32.0	30.0
3.0	0.0	40.0	38.5	—	—	—	—
4.0	0.0	36.0	42.5	—	—	—	—
7.5	11.5	—	47.0	—	—	—	—
10.0	35.0	22.0	—	—	—	—	—
15.0	46.6	—	40.5	—	—	—	—
20.0	—	—	40.0	—	—	—	—
25.0	52.0	—	—	—	—	—	—
30.0	54.0	—	—	—	—	—	—
40.0	55.0	—	—	—	—	—	—

Table IV and Figs. 5 and 6 show that each salt exerts its maximum influence on the amylase formation at its particular salt concentration. The concentration below and above the optimum dosage affects the enzyme yield. Higher salt concentration in general seems to lower the amylase activity excepting in the case of potassium hydrogen

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phosphate. The amylase formation with K_2HPO_4 is negligible within the concentration 0–6 mg, after which it shoots up (see Fig. 6) till the salt concentration has reached 15 mg. The maximum amylase yield is, however, realized when 40 mg of the salt is supplied to 10 ml of the culture medium.

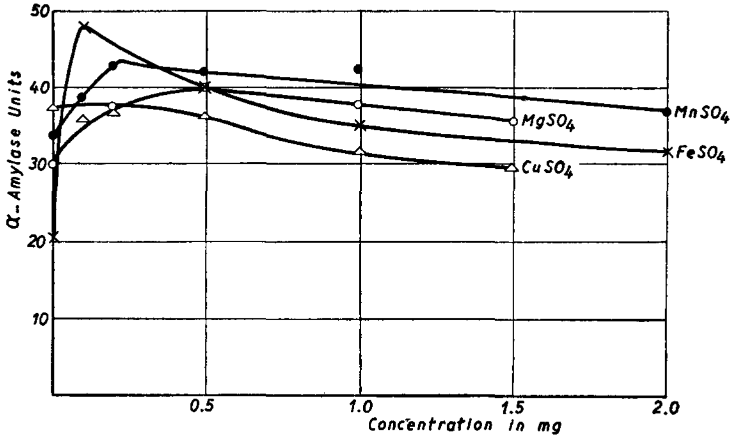

Fig. 5. Influence of the concentration of the salts on the formation of amylase

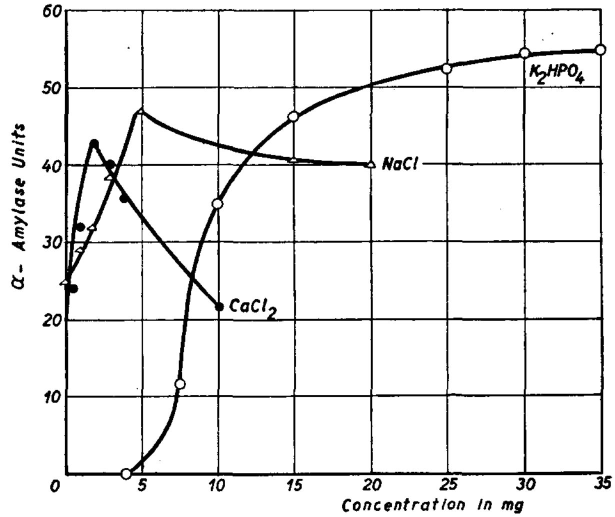

Fig. 6. Influence of the concentration of the salts on the formation of amylase

Trace salts such as $MnSO_4$ and $FeSO_4$ exert their benificial influence when concentrations as low as 0.1 to 0.5 mg are provided in the medium. Copper sulphate did not show any appreciable difference in the amylase activity when the complete Basal Medium was supplemented with different concentrations of the salt.

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SUMMARY

1. The ratio between ammonium nitrate and carbohydrate in the culture medium has been found to exert a definite influence on the amylase secretion of *B. subtilis*, N.C.T.C.; 2027N, and on its growth.

2. Among the various ratios 1:50 is the optimum, giving the maximum amylase yield.

3. Higher concentrations of ammonium nitrate and starch, while maintaining the ratio 1:50, are also found to influence the secretion of amylase. Amounts of 8 mg ammonium nitrate and 400 mg of starch per 10 ml of culture medium give the highest yield of the enzyme.

4. Mineral salts are indispensable for the formation of bacterial amylase. The absence of any of the salts studied results in a decrease in amylase yield.

5. Potassium hydrogen phosphate seems to be very essential, as its absence prevents the bacterial growth.

6. The beneficial influence of salts on the formation of amylase is in the following order:

$$K_2HPO_4 > CaCl_2 > NaCl > FeSO_4 > MgSO_4 > MnSO_4.$$

7. The optimum concentration of different salts for the production of amylase has been determined.

RÉSUMÉ

1. On a trouvé que le rapport nitrate d'ammonium/hydrate de carbone dans le milieu de culture influence d'une façon bien déterminée la sécrétion d'amylase de *B. subtilis*, N.C.T.C., 2027N, ainsi que sa croissance.

2. Parmi les différents rapports, 1:50 est le rapport optimum, donnant lieu au rendement maximum en amylase.

3. Nous avons trouvé que des concentrations en nitrate d'ammonium et en amidon plus élevées, mais dans le même rapport de 1:50, influencent également la sécrétion d'amylase. Des quantités de 8 mg de nitrate d'ammonium et de 400 mg d'amidon par 10 ml de milieu de culture donnent le rendement en enzyme le plus élevé.

4. Les sels minéraux sont indispensables pour la formation de l'amylase bactérienne. L'absence d'un quelconque des sels examinés produit une diminution du rendement en amylase.

5. Il semble que le phosphate de potassium secondaire soit absolument nécessaire, étant donné que son absence empêche la croissance bactérienne.

6. Les avantages de la présence des sels pour la formation de l'amylase peuvent être indiqués par la gradation suivante:

$$K_2HPO_4 > CaCl_2 > NaCl > FeSO_4 > MgSO_4 > MnSO_4.$$

7. La concentration la plus favorable pour la production d'amylase a été déterminée pour différents sels.

ZUSAMMENFASSUNG

1. Es wurde festgestellt, dass das Verhältnis Ammoniumnitrat/Kohlenhydrat im Nährboden einen ganz bestimmten Einfluss auf die Amylasesekretion von *B. subtilis*, N.C.T.C., 2027N, und auf sein Wachstum ausübt.

2. Das vorteilhafteste Verhältnis, das die beste Ausbeute an Amylase gibt, ist 1:50.

3. Höhere Konzentrationen von Ammoniumnitrat und Stärke im selben Verhältnis beeinflussen ebenfalls die Amylasesekretion. 8 mg Ammoniumnitrat und 400 mg Stärke pro 10 ml Nährlösung geben die höchste Ausbeute an Enzym.

4. Mineralsalze sind für die Bildung von Bakterienamylase unbedingt erforderlich. Die Abwesenheit eines der untersuchten Salze hat Verminderung der Ausbeute an Amylase zur Folge.

5. Sekundäres Kaliumphosphat scheint besonders wichtig zu sein, da Fehlen dieses Salzes das Wachstum der Bakterien verhindert.

6. Der günstige Einfluss der Salze auf die Amylasebildung kann durch die folgende Reihe veranschaulicht werden:

$$K_2HPO_4 > CaCl_2 > NaCl > FeSO_4 > MgSO_4 > MnSO_4.$$

7. Die Konzentration, welche für die Amylasebildung am günstigsten ist, wurde für verschiedene Salze bestimmt.

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