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REFERENCES

- Aebi, H. & Abelin, I. (1953). *Biochem. Z.* **324**, 364.
 Blackburn, C. M., McConahey, W. M., Keating, F. R. & Albert, A. (1954). *J. clin. Invest.* **33**, 819.
 Carr, E. A. & Riggs, D. S. (1953). *Biochem. J.* **54**, 217.
 Chappell, J. B. & Perry, S. V. (1954). *Nature, Lond.*, **173**, 1094.
 Deiss, W. P., Albright, E. C. & Larson, F. C. (1953). *Proc. Soc. exp. Biol., N.Y.*, **84**, 513.
 Gross, J. & Pitt-Rivers, R. (1953a). *Biochem. J.* **53**, 645.
 Gross, J. & Pitt-Rivers, R. (1953b). *Biochem. J.* **53**, 652.
 Hoch, F. L. & Lipmann, F. (1953). *Fed. Proc.* **12**, 218.
 Karush, F. (1952). *J. phys. Chem.* **56**, 70.
 Klemperer, H. G. (1955). *Biochem. J.* **60**, 122.
 Lardy, H. A. & Feldott, G. (1951). *Ann. N.Y. Acad. Sci.* **54**, 636.
 Martius, C. & Hess, B. (1951). *Arch. Biochem. Biophys.* **33**, 486.
 Oster, G. & Grimsson, H. (1949). *Arch. Biochem. Biophys.* **24**, 119.
 Price, C. A. & Davies, R. E. (1954). *Biochem. J.* **58**, xvii.
 Rawson, R. W., Rall, J. E., Pearson, O. H., Robbins, J., Poppell, H. F. & West, C. D. (1953). *Amer. J. med. Sci.* **226**, 405.
 Roche, J., Michel, O., Michel, R. & Tata, J. (1954). *Biochim. biophys. Acta*, **13**, 471.
 Taugog, A. & Chaikoff, I. L. (1948). *J. biol. Chem.* **176**, 639.

The Mechanism of Itaconic Acid Formation by *Aspergillus terreus*

1. THE EFFECT OF ACIDITY

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It has been reported that in cultures of *Aspergillus terreus* a substantial accumulation of itaconic acid can only be obtained under acid condition; a pH of about 2 has been found to be the optimum (Lockwood & Reeves, 1945; Moyer & Coghill, 1945; Lockwood & Nelson, 1946). Discussing their results, Lockwood & Reeves (1945) advanced two possible explanations to account for this phenomenon: (1) itaconic acid is produced by the mould only at low pH; (2) itaconic acid is produced over a wide pH range, but is further metabolized at higher pH. In their discussion these authors report the results of an experiment in which they investigated the rate of utilization of itaconic acid by *A. terreus* at different pH values. They found that the higher the starting pH of the culture, the faster the disappearance of itaconic acid. These results do not, however, throw much light upon the problem in question, since it may well be that itaconic acid is preferentially metabolized at higher pH even if it is formed only at low pH.

This paper presents the results of an investigation on the influence of pH on the formation of itaconic acid by *A. terreus*. The results indicate that a low pH is necessary for the formation of the enzyme system producing itaconic acid.

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EXPERIMENTAL

Fermentation experiments. The experiments were carried out with *A. terreus*, strain NRRL 1960. Cultures were grown in 500 ml. flat-bottom flasks containing 100 ml. medium of the following composition: 5 g. glucose monohydrate; 0.3 g. $(\text{NH}_4)_2\text{SO}_4$; 0.3 g. anhydrous CaSO_4 ; 0.05 g. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 0.01 g. KH_2PO_4 ; made up to 100 ml. with tap water. The medium was autoclaved at 10 lb./sq.in. for 10 min., each flask inoculated with 1–2 sq.cm. of a spore mat prepared according to Moyer & Coghill (1945) and incubated on a reciprocating shaker (4 cm. amplitude, 180 oscillations/min.) at 30°. The above medium has a pH of 5.5 at the time of inoculation. During growth of the mycelium the pH decreases to about 2.1, and remains constant at this value during itaconic acid production (Larsen & Hovden, in preparation). For fermentation experiments at pH 6.0 the same procedure as described above was used, but with 1% (w/v) dry sterilized CaCO_3 added aseptically to the autoclaved medium.

Reagents. The glucose monohydrate and anhydrous CaSO_4 used were laboratory reagents from British Drug Houses Ltd. The other chemicals were of analytical grade.

Analytical methods. CO_2 produced during fermentation was determined by absorption in Ascarite, a sodium hydrate-asbestos absorbent (Eimer and Amend, New York). For this purpose a slow stream of CO_2 -free air was drawn through the cultures and absorption tubes. In cultures where CaCO_3 was a constituent of the medium, total CO_2 was determined at the end of the experiment by adding an excess of H_2SO_4 .

The amount of $\text{CaCO}_3\text{-CO}_2$ added was determined either by accurate weighing of the CaCO_3 or by adding an excess of H_2SO_4 to an identically prepared culture flask at the start of the experiment, and the amount so determined was subtracted from the total CO_2 to give the CO_2 produced by fermentation.

Dry weight was determined by filtering the culture through a coarse glass filter, washing the mycelium with boiling water and drying at 110° . The carbon content of the mycelium was determined by the Pregl semimicro dry combustion method. Itaconic acid was determined iodometrically on the macro- and semimicro-scale according to Friedkin (Larsen, 1954); glucose according to Bertrand (Pawlowski, 1932) or Hagedorn-Jensen with the modifications of Fujita-Iwatake (Barrenscheen & Pany, 1941); L-malic and fumaric acids according to Nossal (1952); and succinic acid according to Krebs (Cohen, 1949). Independent estimations of the organic acids were carried out by chromatographic separation on a Celite column as described by Swim & Krampitz (1954). The various fermentation liquors were tested routinely for organic acids by paper chromatography according to Buch, Montgomery & Porter (1952), after removing cations from the solutions with H^+ -charged Dowex 50 resin. pH was determined either with a glass electrode or with pH paper.

Preparation of non-proliferating mycelial suspensions. Considerable difficulty was met in preparing non-proliferating mycelial suspensions which would convert glucose into itaconic acid. The various conventional methods employing centrifugation or filtration resulted in the mycelium losing its ability to produce itaconic acid. However, oxygen was still freely taken up as shown by Warburg experiments. The difficulty was traced back to the sensitivity of the itaconic acid-producing mechanism to lack of oxygen. After a series of trials and failures the following filtering method was successfully adopted:

Six to eight cultures, which were 50–60 hr. old and producing itaconic acid, were transferred, while kept shaken by hand, from the shaking machine to a coarse, sintered glass Büchner filter of 10 cm. diameter. A glass tube, through which a rapid stream of air was blown, was used to stir and aerate the suspension during the filtration. The suspension was filtered as fast as possible by means of suction, great care being taken both to prevent the mycelium from becoming packed on the glass filter plate, and to keep it thoroughly aerated. If the mycelium became packed on the filter the ability to produce itaconic acid was lost. After reducing the volume of the suspension to about 1/5, the original volume was restored by adding tap water containing 0.05% (w/v) $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01% (w/v) KH_2PO_4 and acidified with H_2SO_4 to pH 2.1, and the procedure repeated twice.

The suspension was transferred to a beaker and made up to a volume of about 300 ml. with a tap water medium of the same composition as that used for the washing; aeration was maintained throughout. The suspension was then distributed in portions of 25 ml. into a number of 40 ml. graduated test tubes placed in a water bath at 30° and fitted with glass tubes for constant aeration of the suspensions. Each tube contained approximately 50 mg. wet wt. mycelium/ml. of suspension. Known amounts of glucose were added and samples withdrawn at suitable time intervals for the determination of both residual glucose and the itaconic acid formed. The aeration kept the suspensions in an even motion throughout the experiment. Evaporation from the sus-

pensions was corrected for. A drop of sperm oil was added to each tube to prevent foaming.

For experiments at pH 6 the mycelia were washed on the filter with and suspended in tap water containing 0.05% (w/v) $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.01% (w/v) KH_2PO_4 . 1% (w/v) CaCO_3 was then added to the suspensions to keep the pH constant during acid production. Mycelia grown in the presence of CaCO_3 and later tested for acid production in a non-proliferating state at pH 2.1, were easily washed free of CaCO_3 on the coarse glass filter by the procedure described above.

RESULTS

Fermentation balance in acid cultures (pH 2.1). Preliminary analysis of acid cultures by paper chromatography indicated that itaconic acid was the main organic acid accumulating. A faint spot, indicating and later identified as malic acid, was detected on the paper only when a larger amount of fermentation liquor was analysed. The quantitative determinations of malic acid by both the enzymic and the column chromatographic methods gave the same values, thus showing that malic acid was present as the L-form.

Paper chromatography showed that malic acid appeared at an early stage of the fermentation, before full growth was reached and before the pH had fallen to the low value of 2.1. On the other hand, itaconic acid did not appear until the low pH value was reached.

Table 1. *Fermentation products of glucose by Aspergillus terreus in acid cultures (pH 2.1)*

Volume of culture 100 ml., containing 5000 mg. of glucose at start. At the end of the experiment, after 121 hr. incubation, 4954 mg. of glucose, i.e. 1980 mg. of glucose carbon, had been fermented.

Products formed	Wt. of product/100 ml. culture (mg.)	Product carbon/100 ml. culture (mg.)	Product carbon as % of glucose carbon fermented
Itaconic acid	2320	1072	54.2
L-Malic acid	53	19	1.0
Carbon dioxide	1743	476	24.0
Mycelium (dried)	748*	296	15.0
Total recovery	—	1863	94.2

* The dried preparation of the mycelium had an ash content of 21.0%.

When no more organic acid accumulated in the cultures, as determined by alkali titration, the residual glucose, carbon dioxide, mycelial carbon, itaconic and malic acids were determined. The results of a typical experiment are given in Table 1, and show that practically all the glucose carbon disappearing was recovered in the four products given in the table. Itaconic acid carbon accounts for the larger part of the glucose carbon fermented.

Fermentation balance in cultures buffered with calcium carbonate (pH 6.0). In the presence of an excess calcium carbonate the pH of the cultures was kept constant at 6.0 throughout the fermentation. Paper chromatographic analysis of the fermentation liquor from such cultures showed an accumulation

Table 2. *Fermentation products of glucose by Aspergillus terreus in cultures buffered with CaCO₃ (pH 6.0)*

Volume of culture 100 ml., containing 5000 mg. of glucose at start. At the end of the experiment, after 141 hr. incubation, 4900 mg. of glucose, i.e. 1960 mg. of glucose carbon, had been fermented.

Products formed	Wt. of product/100 ml. culture (mg.)	Product carbon/100 ml. culture (mg.)	Product carbon in % of glucose carbon fermented
L-Malic acid	522	187	9.5
Succinic acid	242	99	5.1
Fumaric acid	36	15	0.8
Carbon dioxide	2565	700	35.7
Mycelium (dried)	3027*	790	40.3
Total recovery	—	1791	91.4

* The dried preparation of the mycelium had an ash content of 45.4%, largely due to CaSO₄ formed by the liberation of CO₂ from CaCO₃ by H₂SO₄.

of malic, succinic and fumaric acids. The malic acid was shown to be present as the L- form. Using the qualitative and quantitative methods described above no itaconic acid was found to accumulate under the conditions of these experiments.

Similar results were obtained in cultures buffered with phosphate, thus indicating that the difference in fermentation pattern from the acid cultures was due to the effect of pH and not to the calcium carbonate itself.

The results of quantitative analyses of a calcium carbonate buffered culture are given in Table 2. Carbon dioxide plus mycelial carbon account for the larger part of the glucose carbon fermented. Organic acids correspondingly account for only 15%.

Itaconic acid-producing ability of non-proliferating mycelia. Itaconic acid-producing cultures of pH 2.1 were filtered and the mycelium washed, suspended and distributed into test tubes as described above. In some of the suspensions pH was adjusted to 2.1 with sulphuric acid, in others to 6.0 with calcium carbonate. Itaconic acid was determined as a function of time both in the presence and in the absence of glucose. The results of the experiment are shown graphically in Fig. 1. In the absence of

Fig. 1. Itaconic acid production from glucose by non-proliferating mycelia grown in an acid environment (pH 2.1). A, Mycelia suspended in acid tap-water solution (pH 2.1); B, mycelia suspended in neutral tap-water solution (pH 6.0). ○—○, Glucose utilization; ●—●, endogenous itaconic acid production; ●—●, itaconic acid production in the presence of glucose.

glucose a substantial production of itaconic acid occurred at both pH values; the rate of itaconic acid production being somewhat higher at the higher pH. The analysis of residual glucose showed that the sugar disappeared at about equal rates at both pH values.

Fig. 2. Itaconic acid production from glucose by mycelia which have been grown at pH 6.0 and then washed and suspended in full growth medium of pH 2.1. ○—○, Glucose utilization; ●—●, itaconic acid production in the presence of glucose.

The molar yields of itaconic acid from glucose after 6 hr. have been calculated from the data of the above experiment on the assumption that one mole of glucose can, theoretically, give rise to one mole of itaconic acid. At pH 2.1 the yield of itaconic acid was 49% and at pH 6.0, 52%. The corresponding yields computed by subtracting the itaconic acid formed endogenously were 30 and 25%, respectively.

The experiment was repeated using cultures grown in the presence of calcium carbonate at pH 6.0. The cultures were filtered, and the mycelium suspended at pH 2.1 and 6.0, as for the acid cultures. Itaconic acid production was followed in the

presence and absence of glucose. It was found that no itaconic acid accumulated either at pH 2.1 or 6.0. Glucose was, however, rapidly consumed at both pH values.

Adaptation to itaconic acid production by growing mycelia. Cultures grown in the presence of calcium carbonate at pH 6.0 were harvested by filtration and suspended in full growth medium containing 3% (w/v) glucose, pH being adjusted to 2.1 with sulphuric acid. Consumption of glucose and accumulation of itaconic acid were followed for 25 hr. The results of the experiments are shown graphically in Fig. 2. Glucose was consumed at about constant rate during the period. No itaconic acid accumulated during the first 5 hr. At the close of the experiment, however, significant amounts of itaconic acid were detected in the reaction tube. Between the 23rd and 25th hour, glucose was converted into itaconic acid with a yield of 28% (mole for mole).

DISCUSSION

The results reported above do not support the view that a low pH favours the accumulation of itaconic acid by *A. terreus* by inhibiting the normal dissimilation of glucose taking place at neutrality. Similarly, the results do not support the contention of Lockwood & Reeves (1945) that itaconic acid is produced over a wide pH range but is consumed faster at higher pH. This conclusion is based upon the fact that non-proliferating mycelia, grown in an acid environment (pH 2.1) will convert glucose into itaconic acid with about the same yield at pH 6.0 and 2.1. Furthermore, non-proliferating mycelia, grown in a neutral environment (pH 6.0), are unable to produce itaconic acid either at pH 6.0 or at 2.1.

However, a mycelium which is grown in a neutral environment and then transferred to an acid environment containing a complete growth medium will gradually develop the ability to produce itaconic acid from glucose. These observations indicate that an acid environment is necessary for the synthesis of an essential enzyme system, or an essential part of such a system, operating in the conversion of glucose into itaconic acid.

SUMMARY

1. The fermentation pattern of *Aspergillus terreus* is different at pH 2.1 and 6.0. At pH 2.1 practically all the glucose carbon fermented is accounted for as itaconic acid, carbon dioxide and mycelium. At pH 6.0 no itaconic acid accumulates, and the glucose carbon is converted into L-malic, succinic and fumaric acids, carbon dioxide and mycelium.

2. Non-proliferating mycelia, grown at pH 2.1, convert glucose into itaconic acid with about equal yields at pH 6.0 and 2.1. Non-proliferating mycelia, grown at pH 6.0, do not accumulate itaconic acid from glucose either at pH 6.0 or 2.1. However, mycelia, grown at pH 6.0 and then transferred to a full growth medium of pH 2.1, will gradually develop the ability to produce itaconic acid.

3. The results indicate that an acid environment is necessary for the formation of an essential enzyme system operating in the conversion of glucose into itaconic acid.

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REFERENCES

- Barrenscheen, H. K. & Pany, J. (1941). *Die Methoden der Fermentforschung*, 1, 1074, ed. Bamann, E. & Myrbäck, K. Leipzig: Georg Thieme.
- Buch, M. L., Montgomery, R. & Porter, W. L. (1952). *Analyt. Chem.* **24**, 489.
- Cohen, P. P. (1949). *Manometric Techniques and Tissue Metabolism*, pp. 166-9, ed. Umbreit, W. W., Burris, R. H. & Stauffer, J. F. Minneapolis: Burgess Publishing Co.
- Larsen, H. (1954). *Methods in Enzymology*, 3, sect. III, ed. Colowick, S. P. & Kaplan, N. O. New York: Academic Press. In press.
- Lockwood, L. B. & Nelson, G. E. N. (1946). *Arch. Biochem.* **10**, 365.
- Lockwood, L. B. & Reeves, M. D. (1945). *Arch. Biochem.* **6**, 455.
- Moyer, A. J. & Coghill, R. D. (1945). *Arch. Biochem.* **7**, 167.
- Nossal, P. M. (1952). *Biochem. J.* **50**, 349.
- Pawlowski, F. (1932). *Die brautechnischen Untersuchungsmethoden*, pp. 126-8. München: R. Oldenbourg.
- Swim, H. E. & Krampitz, L. O. (1954). *J. Bact.* **67**, 419.

The Mechanism of Itaconic Acid Formation by *Aspergillus terreus*

2. THE EFFECT OF SUBSTRATES AND INHIBITORS

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Published work on the itaconic acid fermentation is virtually limited to investigations of the optimum conditions for obtaining high yields of itaconic acid from glucose and sucrose. Very little information is available concerning the conversion of other carbon sources into itaconic acid by mould fermentation. Calam, Oxford & Raistrick (1939) reported that glucose was the only compound, among a number tested, which gave rise to substantial amounts of itaconic acid in surface culture experiments with *Aspergillus terreus*. Citric, malic, pyruvic and acetic acids, either alone or in mixture, gave no or only very small amounts of itaconic acid.

In the present investigation the ability of *A. terreus* to form itaconic acid from a variety of carbon sources has been investigated, using the methods developed in our laboratory for the study of itaconic acid production by cultures growing on a shaker (Larsen & Hovden, in preparation) and by non-proliferating mycelial suspensions (Larsen & Eimhjellen, 1955). The results of these experiments, which have been carried out in both the presence

and absence of metabolic inhibitors, serve as a basis for a discussion of the mechanism of itaconic acid formation.

EXPERIMENTAL

The experiments were carried out with *A. terreus*, strain NRRL 1960. Growth experiments were carried out with cultures grown in acid media on a shaking machine as described in the previous paper (Larsen & Eimhjellen, 1955); using this technique various compounds were tested as carbon source both for growth and for itaconic acid production.

To measure the Q_{O_2} (μ l. O_2 consumed/mg. dry wt. mycelia/hr.) of non-proliferating mycelia the organism was grown on the acid glucose medium (Larsen & Eimhjellen, 1955) at 30° for 50-60 hr., centrifuged and washed with and suspended in either tap water acidified to pH 2.1 with H_2SO_4 or in 0.067 M sodium-potassium phosphate buffer (pH 7.0). Before use the suspensions were aerated for 2 days at 30° to reduce the endogenous respiration. Oxygen uptake was measured in conventional Warburg manometers, and the CO_2 was absorbed by 10% (w/v) KOH in the centre well.

To test for itaconic acid production from various substrates by suspensions of non-proliferating mycelia, 50-60 hr. cultures grown in the acid glucose medium were harvested by filtration as described in the previous paper

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