# STUDIES ON *AEROBACILLUS POLYMYXA*

## IV. FACTORS AFFECTING THE PRODUCTION OF HYDROGENASE AND THE HYDROGENLYASE SYSTEMS

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*Summary*

Once-washed cells of *Aerobacillus polymyxa* harvested from a medium containing peptone, yeast extract, glucose, phosphate, and magnesium sulphate, did not produce hydrogen from formate, glucose, or pyruvate nor was methylene blue reduced in the presence of gaseous hydrogen. Production of hydrogen from formate could be demonstrated in fermenting whole wheat mash and by once-washed cells of *A. polymyxa* grown on strained 10 per cent. wheat mash.

The glucose, peptone, yeast extract medium was found to yield active washed-cell preparations when ferrous sulphate was included in the medium, maximum activity of hydrogenase and of formic, glucose, and pyruvic hydrogenlyases being obtained with approximately 0.05, 0.20, 0.05, and 0.15 per cent. ferrous sulphate respectively. Relatively high concentrations of yeast extract and potassium phosphate were also required for optimum activity.

Media containing suspensions of inert material such as kaolin, calcium carbonate, or paper pulp produced active washed-cell suspensions having different degrees of activity.

Low concentrations of ferrous salts in the medium have been shown to increase the activity of the hydrogenlyase systems in fermenting cultures of *A. polymyxa*. It is suggested, however, that at high concentrations of ferrous salts the insoluble sludge, formed by ferrous ions with phosphate and components of the yeast extract, protects the cells during washing, either from oxidation or from complete leaching out of some essential component of the enzyme systems.

## I. INTRODUCTION

Stickland (1929), Stephenson and Stickland (1932), and Yudkin (1932) have demonstrated the production of hydrogen from formate, glucose, and glycerol by "resting cell" suspensions of *Escherichia coli* and other bacterial species, and Koepsell and Johnson (1942) and Davies (1942) found pyruvate to be a substrate for the production of hydrogen by cells of *Clostridium acetobutylicum*. Investigations by Donker (1926), and by Adams and Stanier (1945), have shown that *A. polymyxa* produces hydrogen and carbon dioxide during the fermentation of media containing glucose or various other mono- or disaccharides.

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In this laboratory, initial attempts to demonstrate production of hydrogen from formate, glucose, or pyruvate by washed-cell suspensions of *A. polymyxa* harvested from a medium containing glucose, peptone, yeast extract, and salts, were unsuccessful, although the production of hydrogen during growth of the cells in such a medium has been demonstrated. The present paper describes methods for the preparation of washed suspensions of *A. polymyxa* showing hydrogenlyase activity.

## II. Methods

Cells of *A. polymyxa* (strain GO(7), Crewther 1950) were harvested by centrifuging 500 ml. of the culture after 20 hr. incubation at 30°C. The cells were washed once in 20 ml. of 0.1M phosphate buffer at pH 6.5, centrifuged, and suspended in 20 ml. of the same buffer solution. Hydrogen evolution was estimated by placing 2-ml. aliquots of cell suspension in the main compartment of Warburg flasks containing 0.5 ml. of substrate solution (1.0M sodium formate, 0.1M glucose, or 0.1M sodium pyruvate at pH 6.5) in the side bulb, and a filter paper roll with 0.4 ml. of 20 per cent. KOH in the centre compartment. The atmosphere was replaced with oxygen-free nitrogen. After equilibration at 30°C. and tipping the flasks, evolution of hydrogen was followed by the usual procedure. Hydrogenase activity was estimated by a similar procedure using an atmosphere of $O_2$-free $H_2$ and 0.5 ml. of 1 per cent. methylene blue in the side bulb as hydrogen acceptor. On tipping the flasks the uptake of molecular hydrogen was measured. Dehydrogenase activities were estimated by the Thunberg technique using methylene blue at a final concentration of 0.02 per cent. Iron was assayed by a modification of the method of Kennedy (1927).

## III. Experimental

In order to determine whether actively fermenting cells of *A. polymyxa* produce hydrogen from formate, 2-ml. aliquots of a well-mixed culture of the organism in 10 per cent. strained wheat mash containing 1 per cent. calcium carbonate were placed in Warburg flasks and the atmosphere replaced with nitrogen. The side bulbs contained 0.5 ml. water or 1M sodium formate, and KOH papers were in the centre cups. The production of hydrogen with time was followed, and, after an initial lag period during which no hydrogen appeared, the rate of hydrogen production in each flask increased to a constant value. The formate or water was then tipped into the culture. The rate of hydrogen production immediately increased to a new constant value in flasks containing formate whereas addition of water had no effect (Fig. 1).

In view of the report by Waring and Werkman (1944) that an adequate supply of iron salts is essential for the production of formic dehydrogenase, hydrogenase, and formic hydrogenlyase by *E. coli*, the effects of adding ferrous sulphate to glucose peptone medium were studied, using the following basal medium:

| | |
|---|---|
| Glucose | 10 g. |
| Difco peptone | 10 g. |
| Difco yeast extract | 5 g. |
| $KH_2PO_4$ | 2 g. |
| $K_2HPO_4$ | 2 g. |
| $MgSO_4.7H_2O$ | 0.25 g. |
| Tap water | 1000 ml. |

$FeSO_4.7H_2O$ was added in amounts up to 3.0 per cent. and cells grown on these media were collected and washed by the standard procedure. Hydrogen evolution by these preparations from formate, glucose, and pyruvate was found to be a function of the amount of ferrous sulphate added to the medium. Figure 2

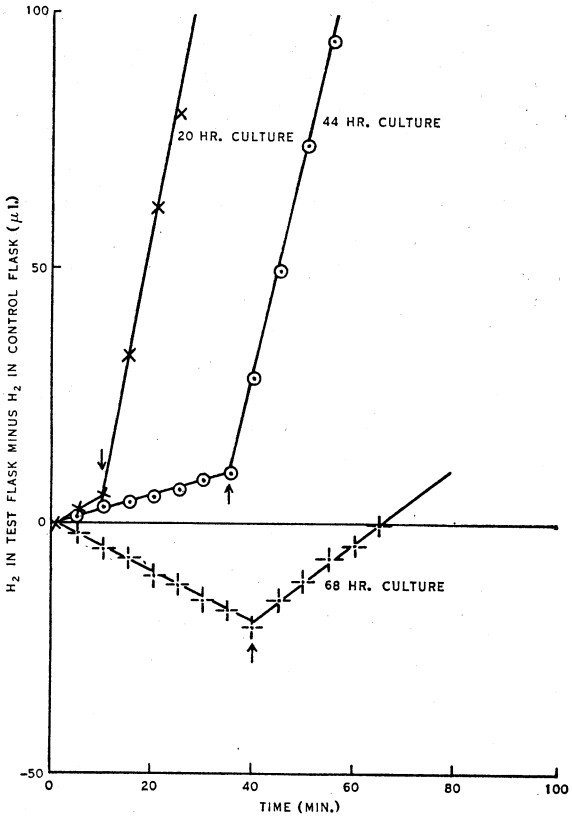


Fig. 1.—Increase in the rate of production of hydrogen on addition of formate to a fermenting wheat mash culture of *A. polymyxa*. Arrows indicate the time of addition of formate to the cultures.

shows that optimal rates of hydrogen production, using formate, glucose, and pyruvate as substrates, were obtained with media containing 0.20, 0.05, and 0.15 per cent. of added $FeSO_4.7H_2O$ respectively. These optimum concentrations were found to vary somewhat in different experiments and with different strains of *A. polymyxa* but were of this order. Viable cell counts and direct

microscopic counts of the cultures and suspensions of *A. polymyxa* showed that changes in cell density were not responsible for the results obtained (Table 1).

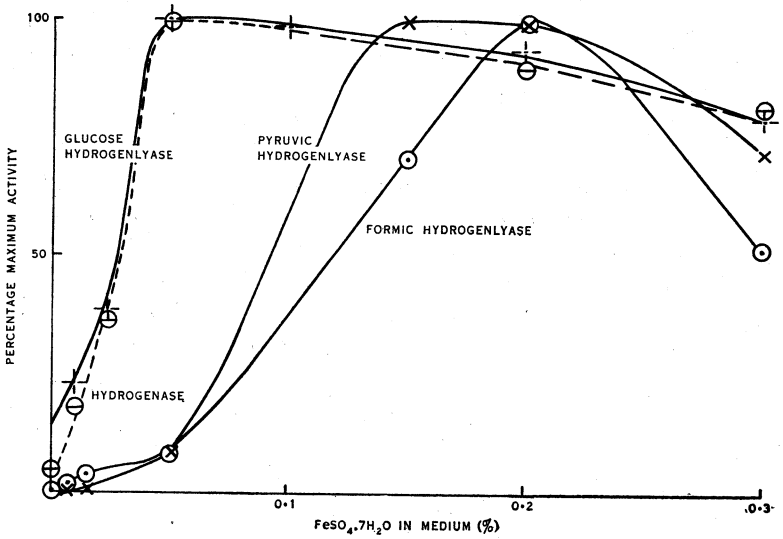


Fig. 2.—Effect of ferrous sulphate in the growth medium on the activity of formic hydrogenlyase and related enzymes of washed-cell suspensions of *A. polymyxa*. Maximum activities against formate, glucose, pyruvate, or hydrogen are in the ratio 100:55:20:70.

In a similar manner it was found that the hydrogenase activity of once-washed cell suspensions varied with the ferrous sulphate content of the medium, optimum activity being obtained at about 0.05 per cent. $FeSO_4.7H_2O$ (Fig. 2). In some experiments it was possible to detect hydrogenase and glucose hydrogenlyase activity in washed cells grown in the absence of added ferrous sulphate.

TABLE 1

SOLUBLE IRON CONTENT OF MEDIA BEFORE AND AFTER GROWTH OF *A. POLYMYXA*

| Total Fe Added (mg./100 ml.) | Viable Cell Count of Culture ($\times 10^{-7}$) | Soluble Fe Before Growth ($\mu$g./100 ml.) | Soluble Fe After Growth ($\mu$g./100 ml.) | $H_2$ Evolved from Formate in 30 Min. (cu. mm.) |
|---|---|---|---|---|
| nil | 60 | 22 | 110 | 0 |
| 0·6 | 9 | 46 | 202 | 2 |
| 2·0 | 5 | 111 | 544 | 4 |
| 6·0 | 5 | 146 | 652 | 7 |
| 20·1 | 4 | 183 | 568 | 17 |
| 60·3 | 5 | 107 | 800 | 135 |

Estimations by the Thunberg technique of the dehydrogenase activity of cells grown on media containing varying amounts of ferrous salts were found

to be misleading, owing to an appreciable slowing of the reduction of methylene blue, which varied in extent with the amount of precipitate. However, washed-cell suspensions of *A. polymyxa* grown on media containing no ferrous sulphate were found to possess an active "glucose dehydrogenase," whereas formic and pyruvic dehydrogenases were not sufficiently active to be detected with certainty by the Thunberg method.

The sludge of insoluble iron salts consisted partly of ferrous hydroxide, partly of basic ferrous phosphate together with an insoluble iron compound of a component of yeast extract. The soluble iron content of the media before and after growth of *A. polymyxa* was determined, the original medium and a 20-hr. culture being filtered through No. 42 Whatman papers for this purpose. The results of these estimations (Table 1) show a considerable increase in soluble iron concentration after growth of bacteria. This is probably due largely to a decrease in pH and possibly in part to the formation of soluble

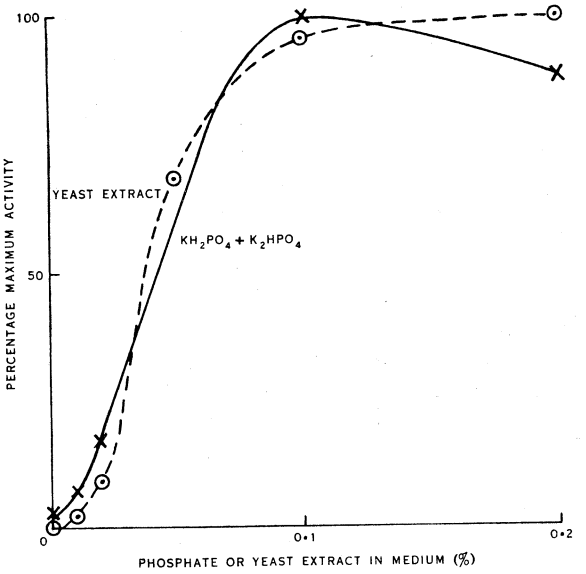


Fig. 3.—Effect of potassium phosphate and yeast extract in the growth medium on the activity of the formic hydrogenlyase of washed cell suspensions of *A. polymyxa*. A mixture of equal proportions of $KH_2PO_4$ and $K_2HPO_4$ was used and the values in the figure refer to the final concentration of each of these salts in the medium.

organic iron compounds by the microorganism. The formation of coordination complexes with fermentation products may also be partly responsible for the increase in soluble iron. Iron salts could not be successfully replaced by salts of other related divalent metals at concentrations that did not inhibit growth. The presence of this sludge, which contained nitrogenous compounds, made it impossible to interpret the results obtained in terms of unit mass of bacteria.

Since both phosphate and yeast extract contributed to the precipitate formed in the medium, experiments were conducted to determine whether they were required for production of hydrogenlyase activity and whether by reducing their concentration higher concentrations of soluble iron could be made available to the organism. The basal medium containing 0.1 per cent. $FeSO_4.7H_2O$ was used for these experiments, the yeast extract concentration being held constant and phosphate varied or vice versa. From Figure 3, it is evident that both yeast extract and phosphate are required for production of active cell suspensions, the optimum concentrations of each being of the order of that used in the original basal medium.

TABLE 2

PROTECTION OF THE FORMIC "HYDROGENLYASE" SYSTEM OF *A. POLYMYXA* BY INSOLUBLE MATERIAL IN THE GROWTH MEDIUM

| *A. polymyxa* Grown on Basal Medium Containing: | $H_2$ Evolved in 30 Min. (cu. mm.) |
|---|---|
| No addition .. .. .. | 0 |
| $FeSO_4.7H_2O$ 0·2% .. .. | 51 |
| $KAl(SO_4)_2H_2O$ 0·1% .. | 0 |
| $CaCO_3$ 1·0% .. .. .. | 14 |
| Kaolin 1·0% .. .. .. | 55 |
| Pulped filter paper 1·0% .. | 5 |

Attempts were made to replace the yeast extract of the basal medium with up to 2 μg./ml. nicotinic acid, nicotinamide, riboflavin, folic acid, pyridoxine hydrochloride, thiamine hydrochloride, sodium pantothenate, pyridoxamine, pyridoxal, and biotin, both singly and collectively. In some experiments pyridoxal and pyridoxamine increased the activity of the washed suspensions to some extent, but in no case did the suspensions approach the activity of cells grown in the presence of yeast extract. Glutamate, glutamine, and asparagine were also without effect in the absence of yeast extract. It seems likely that the action of yeast extract is chiefly one of increasing the cell count in the final culture rather than a specific increase in the hydrogenlyase activity per unit mass of bacteria, the addition of yeast extract to a glucose-peptone medium containing 0.2 per cent. $FeSO_4.7H_2O$ causing increases in the viable cell count of up to 100-fold. Smaller increases in count were observed in media containing pyridoxamine and pyridoxal.

In view of the fact that strained wheat mash and the ferrous sulphate medium which produced active cell suspensions of *A. polymyxa* both contained some form of sediment, six flasks were prepared containing the basal medium with the addition of 0.01 per cent. $FeSO_4.7H_2O$ (normally insufficient to give a washed-cell suspension producing hydrogen from formate), to five of which additions of 1 per cent. $CaCO_3$, 1 per cent. acid-washed kaolin, 1 per cent. pulped filter paper, 0.1 per cent. $KAl(SO_4)_2.H_2O$, or 0.2 per cent.

$FeSO_4.7H_2O$ were made. After inoculation, incubation, and collection by the usual procedure it was found that active washed suspensions were obtained from the flasks containing calcium carbonate or kaolin, particularly the latter. Some slight activity was also obtained in the preparation containing filter paper (Table 2). The bacteria were largely enveloped in a mixture of the sediment and slime, which necessarily reduced the effectiveness of washing.

In order to evaluate the actual increase in the activity of the enzyme system responsible for the evolution of hydrogen from formate due to the incorporation of $FeSO_4.7H_2O$ in the growth medium, the rate of production of hydrogen from 2-ml. aliquots of fermenting basal medium containing varying amounts of $FeSO_4.7H_2O$ was determined before and after addition of formate.

As in the initial experiments of this type, the rate of production of hydrogen was followed until it became constant, the substrate was then added and the increased constant rate of hydrogen evolution measured. Figure 4 indicates that cultures containing 0.003 per cent. $FeSO_4.7H_2O$ possessed maximum formic hydrogenlyase activity. Concentrations of the order of 0.2 per cent. $FeSO_4.7H_2O$ inhibited the production of hydrogen.

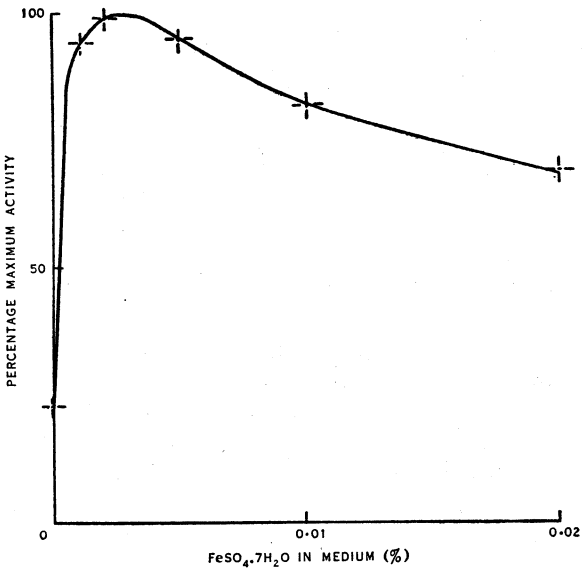


Fig. 4.—Effect of ferrous sulphate in the medium on the rate of production of hydrogen by a fermenting culture of *A. polymyxa* on addition of formate. Sodium formate solution was added to 2-ml. aliquots of the culture after a constant rate of hydrogen production had been reached and the final constant rate of evolution was measured.

## IV. Discussion

The results shown in Figure 4 indicate that addition of small amounts of ferrous sulphate to the basal medium increases the activity of the hydrogenlyase and hydrogenase systems in a fermenting culture of *A. polymyxa*, whereas

higher concentrations actually decrease the production of these enzymes or inhibit their activity. The role of high concentrations of ferrous salts in producing active washed-cell suspensions appears to be the formation of a sludge of insoluble iron salts which protects the enzyme system during the process of collection and washing of the bacteria. The production of a copious polysaccharide slime by *A. polymyxa* undoubtedly assists such a protective mechanism and makes difficult the thorough washing and dispersion of the bacteria. This view is substantiated by the production of active washed-cell suspensions from media containing kaolin or calcium carbonate.

These results appear to be related to those of Yudkin (1932) who found that, although a particular strain of *E. coli* produced hydrogen during the fermentation of a glucose medium with an inorganic nitrogen source, washed-cell suspensions from this medium failed to produce hydrogen from formate or glucose. Yudkin attributed this to the washing out of some stabilizing substance in the medium.

It is also noteworthy that whereas formic and pyruvic hydrogenlyases required the addition of approximately 0.2 per cent. ferrous sulphate to the medium to ensure optimal activity of washed suspensions, 0.05 per cent. $FeSO_4.7H_2O$ was sufficient to produce a protective sludge with glucose hydrogenlyase and hydrogenase. Glucose dehydrogenase did not require protection during washing whereas formic and pyruvic dehydrogenase activities were absent from washed cells obtained from media containing no added iron salts. All three dehydrogenases have been demonstrated in washed-cell suspensions from media containing kaolin, and it seems probable therefore that the dehydrogenases form an integral part of the hydrogenlyase systems. The evidence for this view is not sufficient to disprove Stephenson and Stickland's suggestion that the hydrogenlyases are distinct from the dehydrogenases and hydrogenase (1932), but suggests a reinvestigation of the problem.

## V. Acknowledgments

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## VI. References

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