

## The Effect of Agitation on the Rate of Acid Formation by *Lactobacillus delbrueckii*\*

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*Summary.* The effect of agitation on the rate of acid formation by the microaerophilic organism *Lactobacillus delbrueckii* was studied. Fermentations were conducted at constant temperature and pH, and with the continuous addition of a nutilite source. The range of agitator speeds was from 85 to 720 rev/min.

The acid formation rate was found to increase with increasing agitator speeds and decrease with decreasing speeds if corn-steep liquor was the nutilite source. No effect of agitation was observed with yeast extract as the nutilite source.

The data from corn-steep liquor media were found to be consistent with a mass transfer mechanism, but did not warrant a conclusion as to the specific nature of the effect of agitation. It was concluded, however, that the effect of agitation on the acid formation rate was at most very small.

### Introduction

This work was undertaken to study quantitatively the effect of mechanical agitation on the rate of lactic acid formation by the homofermentative organism *Lactobacillus delbrueckii*.

Numerous references on the effects of agitation (and aeration) on the rates and yields of aerobic fermentations may be found in the literature. The comprehensive review by Finn<sup>1</sup> indicates the extensive interest in this subject. There is very little

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published work, however, on the effects of agitation on anaerobic and microaerophilic organisms.

Rogers and Whittier,<sup>2</sup> and Rahn *et al.*<sup>3</sup> have reported on the effects of stirring on the growth of the facultative anaerobe *Streptococcus lactis*. The former reported that mechanical agitation, bubbling air, and bubbling nitrogen produced, in that order, higher cell populations than were obtained in unstirred cultures. The latter reported that agitation with air or oxygen decreased the fermentation rate, while agitation with nitrogen increased the rate, compared with unstirred cultures. Cutter<sup>4</sup> compared fermentations at different rates of mechanical agitation and concluded that the only apparent effect of agitation on the anaerobic fermentation of glucose by yeast was to decrease the length of the lag phase.

In view of the scant information on non-aerobic fermentations, this work was initiated to study the effect of agitation on the rate of fermentation by a microaerophilic organism. *L. delbrueckii* was selected for this investigation for several reasons. First, this organism is considered to be microaerophilic,<sup>5</sup> that is, some oxygen is required for growth, but the organism prefers a lower oxygen tension than that when exposed to the air;<sup>6, 7</sup> hence, it was not necessary to bubble air through the media. Certain results to be cited later in this paper indicate that *L. delbrueckii* NRRL B-445 is indifferent to the oxygen concentration. Secondly, considerable work has been done with this organism, and a convenient and accurate method has been developed for determining the fermentation rate.<sup>8</sup> Thirdly, it has been observed that under properly controlled conditions, the total rate of acid formation in the fermentor could be maintained at a constant value for a considerable period of time.<sup>9, 10</sup>

In order to obtain a constant rate, the temperature and pH were closely controlled, the agitator speed was held constant, and a source of nutrient materials was added to the fermenting medium essentially continuously throughout the course of a fermentation.

This period of constant fermentation rate is particularly valuable, since it allows the observation of the effect of a change of a single variable on the rate of fermentation during the course of a single fermentation.

### Equipment and Methods

The equipment employed was the same as that described by Gillies and Kempe,<sup>11</sup> but modified to allow vigorous agitation and variation of the agitator speed. Agitation was accomplished with a vaned-disc impeller driven through a belt-and-pulley

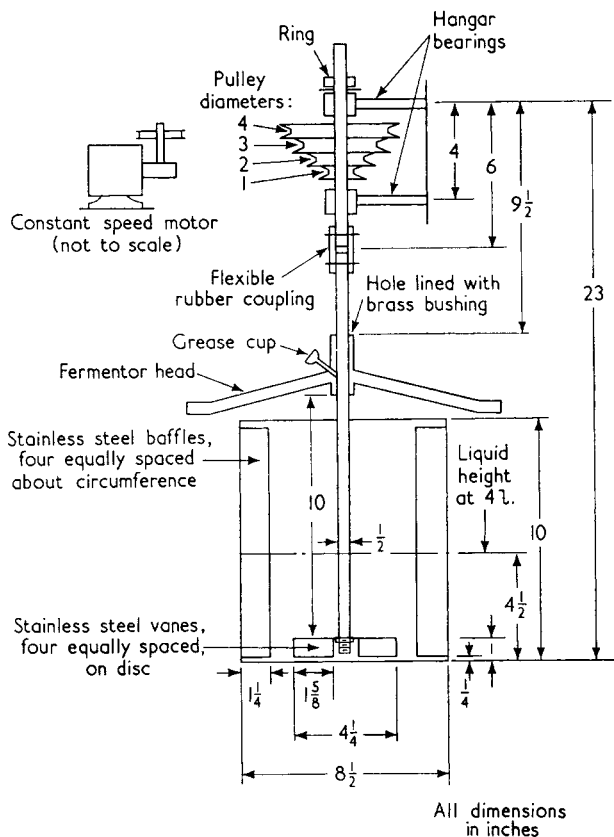


Fig. 1. Details of fermenter interior and agitation system

system by a  $\frac{1}{20}$  h.p. motor. The details of the fermenter interior and agitation system are shown in Fig. 1. The agitation system was such that the agitator speed could be varied from 85 to 720 rev/min. Four stainless steel baffles equally spaced about the periphery of the vessel provided sufficient baffling so that

vortexing did not occur, even at the highest agitator speeds employed.

The temperature of the medium was controlled within  $\pm 0.2^{\circ}\text{C}$  by two electric heaters in a water bath surrounding the fermentor. One heater was actuated by a bi-metallic thermo-regulator in the bath and the other by a mercury thermoregulator in the fermentor. The pH was controlled within  $\pm 0.03$  units by a Beckman Model RX pH meter and a Bristol Pyromaster potentiometer.

Acid formed by the organisms was neutralized with 2N sodium hydroxide, pumped by a Sigmamotor pump\* whenever the pH fell below the control limit. The neutralizing solution was stored in a 1 l. graduated cylinder and the volume of solution remaining was recorded on a revolving kymograph drum. The resulting curve of alkali volume *versus* time was graphically differentiated to determine the instantaneous rate of addition of alkali solution. The total rate of acid formation in the fermentor was directly proportional to the measured rate of alkali addition. Instantaneous acid formation rates were determined with a precision of  $\pm 1$  mequiv/h. It was found that 95 per cent of the titrable acid was lactic (as determined by the method of Friedmann and Graeser<sup>12</sup>). It should be noted that acid formation rates calculated from the rates of alkali addition were total rates, not rates per unit volume. It was the total rate which remained constant in control runs.

The basal medium was the same as that described previously,<sup>11</sup> except that 150 or 180 g of glucose were used. Either corn-steep liquor or yeast extract was used as a source of required and stimulatory nutrilites. The corn-steep liquor used in this work was treated prior to sterilization by the method described by Liggett and Koffler.<sup>13</sup> A quantity of the nutrilitite source was added to the basal medium and additional nutrilitites were added semi-continuously throughout the course of the fermentation by the Sigmamotor pump.<sup>10</sup> Since the pump operated approximately 15 sec of each 2 min when a fermentation was progressing actively, the nutrilitite addition was essentially continuous. It was this continuous addition of nutrilitites which allowed the establishment of a constant rate of acid formation.

\* Sigmamotor Inc., Middleport, New York.

*L. delbrueckii* NRRL B-445 was used throughout the investigation. This organism is classified as microaerophilic.<sup>5</sup>

The experimental procedure followed that described earlier,<sup>10, 11</sup> except that the agitator speed was changed periodically during a single fermentation by varying the pulley diameter ratio. The temperature and pH were controlled at the same level throughout a run. The procedure of changing the agitator speed took less than 5 min, so no significant changes in the medium occurred during this operation. A period of 4 to 6 h at each agitator speed was allowed so that a constant rate would be established. Only three or four different agitator speeds could be used during a single run, since the duration of the constant rate period was usually about 20 h.

### Results

During the course of this work it was repeatedly found, in accordance with the observations of Gillies,<sup>9</sup> that a constant rate of acid formation was established by *L. delbrueckii* if the temperature, pH, and agitator speed were held constant and nutilites were added continuously. This constant rate was observed both in corn-steep liquor and yeast extract media.

In corn-steep liquor media the acid formation rate changed measurably with changes in agitator speed; in yeast extract media this effect was not observed. It should be noted that all rates under discussion are total rates, not rates per unit volume.

Fig. 2 shows the results of a typical run in a corn-steep liquor medium. The acid formation rate, in mequiv/h, is plotted as a function of the time after inoculation; periods at various agitator speeds are indicated by horizontal arrows. Fig. 2 shows that when the agitator speed was increased from 85 to 720 rev/min, the acid formation rate increased from 49.5 to 55 mequiv/h. A decrease in agitator speed from 720 to 275 rev/min was followed by a decrease in rate from 55 to 53 mequiv/h. When the speed was returned to the original value of 85 rev/min the rate returned to its original value of 49.5 mequiv/h. These results illustrate that each change in agitator speed in the range investigated resulted in a rate change. Furthermore, the effect of agitation was reversible, i.e. upon returning the speed to a previous value after

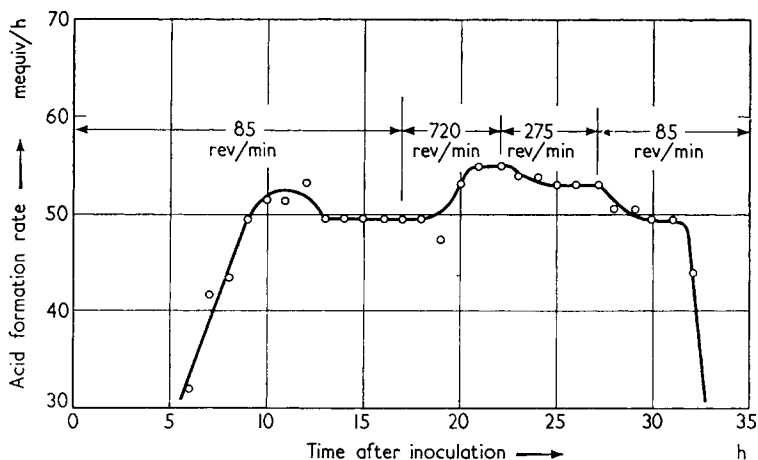


Fig. 2. Effect of agitator speed on the rate of acid formation by *L. delbrueckii*, in a glucose-corn-steep liquor medium at 43.4°C and pH 5.40

a change, the corresponding previous rate was established. It is also interesting to note that when the agitator speed was changed a new constant rate was not established for a period of 2 to 4 h, although a change in rate was noticed almost immediately.

Several runs were made with corn-steep liquor media and the aforementioned results were consistently observed and were reproducible. The results from several runs are summarized in Table I.

Table I. The effect of agitator speed on the rate of acid formation by *L. delbrueckii* in glucose-corn-steep liquor media at 43.4°C

Run	pH	Agitator speed, rev/min	Rate of acid formation, mequiv/h
24	5.45	85	49
		550	56
25	5.40	85	49.5
		720	55
		275	53
26	5.53	85	56.5
		275	60
		550	67
27	5.40	85	53
		275	58.5
		430	65.5

In media employing yeast extract as the nutrilitie source there was no consistent effect of agitation. Fig. 3 shows the results of one run wherein yeast extract was used. Examination of Fig. 3 shows no effect attributable to agitation. When the agitator speed was changed from 85 to 170 rev/min the rate had not yet become constant, but did establish a constant value at 170 rev/min. An increase in agitator speed to 320 rev/min caused no change in rate for about 5 h, at which time slight increase occurred. After the rate had increased at 320 rev/min a change to 85 rev/min caused no further rate change. This plot is representative of the

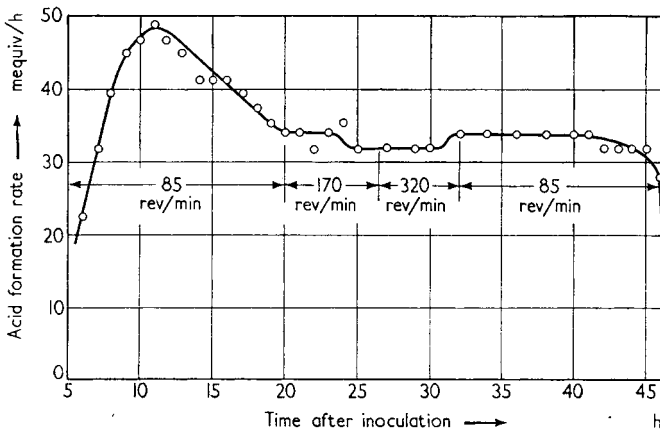


Fig. 3. Effect of agitator speed on the rate of acid formation by *L. delbrueckii*, in a glucose-yeast extract medium at 43.5°C and pH 5.60

results obtained in yeast extract media, in that no consistent or reproducible effect of agitation was observed. Furthermore, the changes which sometimes did occur were small in magnitude. It was concluded that agitation had no measurable effect on the fermentation rate in yeast extract media.

A possible mechanism for the observed effect of agitation in corn-steep liquor media is a change in the liquid phase mass transfer coefficient (see discussion). In accordance with this mechanism, the acid formation rate should be proportional to a liquid film mass transfer coefficient, the total organism-liquid interfacial area, and a driving force given by the difference

between the bulk and interfacial concentrations of a rate-limiting material, according to the equation,

$$r = k_L A(C - C_i) \quad (1)$$

where  $r$  is the acid formation rate,  $k_L$  is a liquid film mass transfer coefficient,  $A$  is the total organism-liquid interfacial area, and  $C$  and  $C_i$  are the bulk and interfacial concentrations of a rate-limiting material, respectively.

Empirical correlations for solid-liquid mass transfer coefficients in agitated vessels indicate that the mass transfer coefficient should vary exponentially with the agitator speed if the vessel and impeller dimensions and physical properties of the fluid and solid remain constant,<sup>14, 15</sup> thus,

$$k_L = k_1(R)^a \quad (2)$$

where  $k_1$  is a constant for a given set of experimental conditions,  $R$  is the agitator speed, and  $a$  is a constant.

If it is assumed that the interfacial area and driving force (or their product) remain constant when the agitator speed changes, then equations (1) and (2) indicate that the acid formation rate should vary exponentially with agitator speed, thus,

$$r = k_2(R)^a \quad (3)$$

where  $k_2$  is a constant for a given set of experimental conditions. Note that the constant  $k_2$  is a function of the dimensions of the vessel and impeller, the physical properties of the fluid and solid, the interfacial area, the medium composition, and the temperature and pH.

According to the postulated mechanism and aforementioned assumptions, a plot of the acid formation rate *versus* the agitator speed should be linear on logarithmic co-ordinates. When the data of Table I were plotted on logarithmic co-ordinates it was found that the points for each run scattered about a different straight line. However, each line had approximately the same slope (equal to the exponent,  $a$ ), indicating that the constant  $k_2$  was different for each run.

Because of the few points available for any single run, the data were re-plotted so that a single line should fit the data for all the runs. Since for any one run  $k_2$  should be constant, if the ratio of



the acid formation rate at one agitator speed to that at another is plotted against the corresponding agitator speed ratio on logarithmic co-ordinates, a straight line should result with slope  $a$ , as shown by the equation

$$\frac{r_1}{r_2} = \left( \frac{R_1}{R_2} \right)^a \quad (4)$$

where  $r_1$  and  $r_2$  are the acid formation rates at agitator speeds  $R_1$  and  $R_2$ , respectively. Equation (4) should apply to all runs, since the coefficient  $k_2$  does not appear.

Fig. 4 is a plot of the ratio of acid formation rates *versus* the ratio of the corresponding agitator speeds on logarithmic co-ordinates. Fig. 4 shows that there is considerable scatter in the

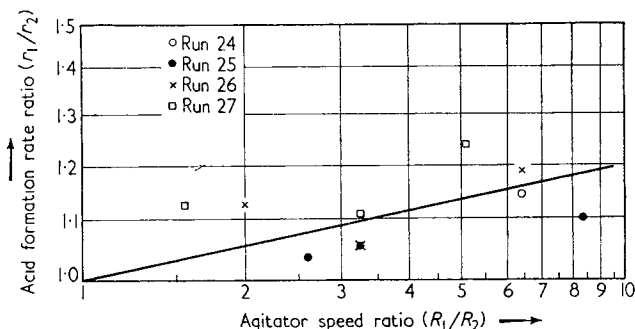


Fig. 4. Ratio of acid formation rates by *L. delbrueckii* as a function of the corresponding ratio of agitator speeds in glucose-corn-steep liquor media.

data, but that the points follow the trend of the 'least-square' line shown on the plot. A  $t$  test at the 95 per cent confidence level showed that the 'least-square' line could pass through the point (one, one) as would be expected from the results shown in Fig. 2. The slope of the line in Fig. 4 was found by 'least-squares' to be 0.08, hence equation (4) becomes

$$\frac{r_1}{r_2} = \left( \frac{R_1}{R_2} \right)^{0.08}$$

Although *L. delbrueckii* is classified as microaerophilic, certain observations during this work indicate that the particular organism used was not sensitive to the oxygen concentration. For

example, a uniform number and size of colonies was observed throughout rolled Prickett tubes containing a freshly sterilized medium composed of 0.5 per cent glucose, 2 per cent yeast extract, and 1 per cent agar. Since the medium should have been nearly devoid of oxygen after sterilization, oxygen would be supplied only by diffusion through the agar. There would then be an oxygen concentration gradient from the top to the bottom of the tubes. If the oxygen concentration had an appreciable effect on the growth of this organism, a gradient in the number and/or size of colonies should have been observed. The absence of such a result therefore indicates little or no effect of oxygen concentration.

The absence of a noticeable effect of oxygen was also observed in stab cultures in the same medium as used for the rolled tubes, and in Petri plates as well. The medium used in the plating work was composed of 2 per cent yeast extract, 2 per cent agar, and 1 per cent glucose. Furthermore, the fact that the organism grew well in the fermentor in liquid media containing either corn-steep liquor or yeast extract without an inert gas blanket demonstrates that atmospheric oxygen does not markedly inhibit its growth in these media.

### Discussion

It seems worth while to consider the significance of the constant rate of acid formation that developed in fermentations continuously supplied with nitrilites. In addition to this result, it was observed that if the same amount of nitrilite source was added initially to the basal medium and none was added continuously, the rate increased to a maximum and then steadily decreased throughout the fermentation.<sup>10</sup> A comparison suggests that some component or components of the nitrilite source limited the fermentation rate under the experimental conditions. That is, the rate-limiting material was disappearing from the medium as the fermentation progressed, either by being consumed by the organism or by some reaction in the medium. When the nitrilite was added at a rate equal to its rate of disappearance, its concentration, and hence the rate of acid formation, remained constant. Thus, it appears possible that liquid phase mass

transfer of this material could be a rate-limiting step in the fermentation process.

In the presentation of the results, an analysis based on a postulated liquid phase mass transfer mechanism was presented in some detail. It was shown that the data obtained in corn-steep liquor media follow a trend consistent with that analysis. This certainly does not prove that the postulated mechanism is correct. In fact, several other mechanisms for the observed phenomenon can be considered.

The effects of agitation should be on the physical condition of the organism or medium, with perhaps a concomitant chemical change. Possible mechanisms include the following: (1) a breaking of chains of organisms into single cells or smaller chains, hence producing more interfacial area; (2) a change in the physical nature of the cell wall due to abrasion; (3) the reduction of gross composition gradients in the medium; (4) an increase in the rate of liquid phase mass transfer to the cells; and (5) an increase in the gas-liquid mass transfer rate.

The first three mechanisms suggested above are not consistent with the observation that agitation had no effect in yeast extract media. If the effect was due to a physical change in the cells or medium it should have occurred in both media. A similar argument would apply to the fourth suggested mechanism, unless the nature of the rate-limiting material was different in the two media. In view of the known differences in composition of yeast extract and corn-steep liquor,<sup>13, 16</sup> it is reasonable to suspect that the rate-limiting material was not the same in these two nutrient source materials.

It should be recognized that objections to a liquid phase mass transfer mechanism have been proposed. Finn has argued that at most only a very small diffusional resistance should exist around single cells of micro-organisms, due to their small size; and, further, that it is unlikely that mechanical agitation could develop a sufficient relative velocity between the cells and the liquid to markedly change that resistance.<sup>1</sup> While his discussion applied specifically to oxygen uptake, the same argument could be extended to a non-aerobic fermentation. There is insufficient experimental evidence currently available, however, to apply this argument critically to the present fermentation.

The fifth mechanism suggested above also remains a possibility. The effect of agitation could have been to change the rate of desorption of dissolved gases (especially carbon dioxide), or of absorption of gases from the atmosphere above the liquid in the fermentor (in particular oxygen). It is quite unlikely, however, that the desorption of carbon dioxide could be a rate-limiting factor, for again, the effect of agitation should have been the same in both media.

The medium was not blanketed with an inert gas as has been the practice of some investigators.<sup>8</sup> Thus, there exists the possibility that oxygen was being dissolved from the air in the fermentor, and that the rate of dissolution increased with increasing agitator speed. However, this mechanism could account for the observations only if the oxygen concentration in the medium was less than the saturation value and: (1) oxygen directly affected the rate of acid formation by the organism, or (2) oxygen affected the medium composition such that the rate of acid formation would be altered. Again, since agitation did not have the same effect in the two media used, the first of these two possibilities is unlikely. The second remains a possibility, however, if oxygen reacted differently with the two nutrilitite sources.

The medium should have been saturated or nearly saturated with oxygen a few hours after inoculation, due to dissolution at the air-liquid interface, unless the organism or the medium exerted an appreciable oxygen demand. Since the organism is classified as microaerophilic and certain results of this work suggest that it is indifferent to oxygen, it should not exert any appreciable oxygen demand. The possibility still remains that the medium might react with dissolved oxygen and thereby exert an 'oxygen demand'. Whether or not the composition of either medium was influenced by oxygen absorption and whether that would affect the rate of acid formation is problematical.

Longsworth and MacInnes<sup>17</sup> have reported that a relatively large increase in the oxygen tension decreased slightly the rate of acid formation by *L. acidophilus*. Such a reduction is consistent with the microaerophilic nature of *L. acidophilus*.<sup>5</sup> That is, a microaerophilic organism should prefer a reduced oxygen tension.<sup>6, 7</sup> Since it was observed that the rate of fermentation by

*L. delbrueckii* increased with increasing agitator speed, the oxygen absorption mechanism is not consistent with the results of Longworth and MacInnes.

In view of the foregoing discussion, it is apparent that there is not sufficient information to specifically determine the mechanism of the effect of agitation on this fermentation. However, the results obtained in corn-steep liquor media are consistent with a liquid phase mass transfer mechanism. In any event, it can be stated that the effect is at most small and for most purposes probably negligible.

### Conclusions

A consistent and reproducible, but small, effect of agitator speed on the rate of acid formation by *L. delbrueckii* was observed in corn-steep liquor media, but not in yeast extract media. The data are consistent with a liquid phase mass transfer mechanism, but do not warrant a conclusion concerning the mechanism.

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