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Aeration-Agitation Studies on the Rifamycin Fermentation

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Summary

Aeration and agitation conditions in 250 l. baffled fermenters with different impeller diameters, speeds, and air flows were studied during fermentations for the production of rifamycin, a new antibiotic produced by *Streptomyces mediterranei*. Dissolved oxygen concentration was continuously measured and the courses of power input, oxygen diffusion rate QO_2, dry weight, viscosity, pH, sugar utilization, and antibiotic titer determined. Higher antibiotic yields occurred when the oxygen demand of the culture was satisfied and an excess of dissolved oxygen was still present during a critical period of the fermentation between the 50th and the 80th hr. To meet this requirement a power input of about 3.0 w./l. and an air flow ranging from 0.8 to 1.5 l./l./min. were found to be necessary.

INTRODUCTION

Rifamycin, a new antibiotic isolated by Sensi et al.,[1] is produced by the fermentation of the *Streptomyces mediterranei sp.* Previous experiments had pointed out the great importance of agitation and aeration in relation to the antibiotic's production. The influence of the mechanical and geometrical characteristics of the fermenter on the efficiency of the aeration and the importance of the latter on the production have been studied.

EQUIPMENT AND METHODS

Fermentation Tank

Stainless steel fermenters with a total capacity of 400 l. and a working capacity of 250 l. have been employed for all tests. The essential characteristics of the fermenter are given in Figure 1. The other important data which characterize it are:

(*1*) The ratio of the height of the fluid to the fermenter diameter is 1.3.

(*2*) Four baffles placed at a 90° angle and having a length equal to 0.10 of the tank diameter.

(*3*) A turbine impeller with 6 straight blades with the following ratios: Impeller diameter/blade length = 4, blade length/blade width = 1.26.

Four different impeller diameters were used for the tests described here: 32, 38, 49, and 59% of the fermenter's internal diameter. The air flow was maintained at a level just inferior to the flooding point, that is, at conditions in which the capacity of the turbine for oxygen diffusion is at a maximum.

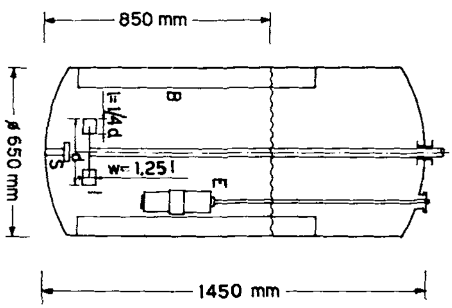

Fig. 1. Fermenter design. S, sparger; I, impeller; B, baffles; E, oxygen electrode; d, impeller diameter; l, blade length; and w, blade width.

Under these conditions the air flow varies from 0.8 to 1.5 v/v per min. according to the turbine diameter and the impeller rotational speed employed.

Figure 2 shows the effect of air flow on power absorbed by the fluid with a standard turbine (250 mm. diameter) at various impeller speeds. The arrows indicate the air flows chosen afterwards in the fermentations performed at different speeds. Similar diagrams have been obtained with the other impellers. The pressure in the tank was kept constant at 0.2 kg./cm.^{2}.

The power drawn by the impeller for the mechanical agitation of the liquid was calculated from measurements of absorbed electrical power and from the diagrams of the mechanical characteristics of the electrical motors employed.

The rheological behavior of samples of fermentation broths drawn from the fermentation tank has been studied with a Mod. KFV Brookfield Viscometer according to the technique suggested by Deindoerfer and West.[2]

The quantity of oxygen dissolved in the fermentation broths has been determined by an amperometric method developed by Gualandi et al.[3] The instrument allowed continuous measurement and recording of the oxygen concentration. The polarographic electrode was plugged inside the fermenter and sterilized together with the medium.

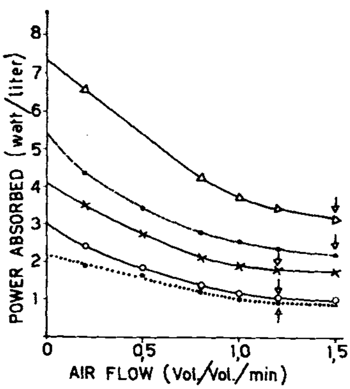

Fig. 2. The effect of air flow on power absorbed at various impeller speeds. Diameter of the impeller: 250 mm.; speed of the impeller: △——△, 500 r.p.m.; ●---●.,400 r.p.m.; X——X, 375 r.p.m.; O——O, 350 r.p.m.; ●····● 275 r.p.m.

Respiration measurements have been taken directly from the fermentation broth samples with Warburg's manometric technique. A control method, and also an alternate technique, consists of measuring the oxygen uptake during the actual conditions of fermentation and determining, by a polarographic method, the quantity of residual O_2 in the air flowing out of the fermenter, while measuring the air flow at the same time.

Fermentation

A master stock of *Streptomyces mediterranei* served to inoculate a seed medium having, before sterilization, the following composition (per liter):

Meat extract	5 g.
Peptone	5 g.
Yeast autolysate	5 g.
Cerelose	20 g.
pH after sterilization	6.2–6.4

Composition of fermentation medium (per liter):

C.S.L. 50% solids	11 g.
$(NH_4)_2SO_4$	14 g.
Cerelose	100 g.
$CaCO_3$	13 g.
pH after sterilization	6.2–6.4
Antifoam	Silicone "A" 2% in vaseline oil

Fermentation temperature in both phases was 28°C. The fermentation tank was inoculated with 5% *v/v* of seed medium grown for 45–48 hr.

The average length of the fermentation was between 118 and 120 hr.

All tests reported in this work have been performed in triplicate. The typical course of a rifamycin fermentation is shown in Figure 3.

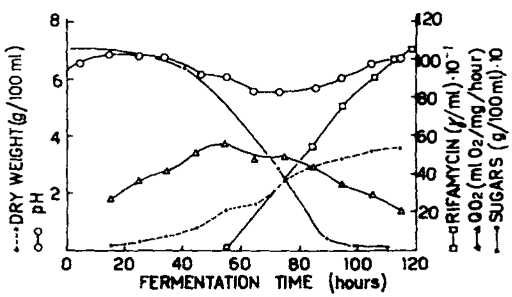

Fig. 3. Characteristics of a typical rifamycin fermentation.

Chemical Analyses

The antibiotic concentration was determined by the spectrophotometric method described by Gallo et al.[4]

The measurement of the sugar content was performed according to the Somögiy method.[5]

The determination of the mycelial dry weight was carried out in the following manner. A precisely measured fraction of the sample was centrifuged and the fluid was discarded. The solid residue was first treated three times with HCl $N/5$ to eliminate all traces of $CaCO_3$ and then washed with a volume of water about twice that of the combined HCl solution. The dry residue obtained on a filter paper was placed in an oven at 105 °C. for 24 hr.

RESULTS

1. Relationship between Power Absorbed and Geometric Characteristics of the Impellers

The curves representing the mechanical power absorbed by liquid media have been obtained from absorption tests with different diameter impellers.

These curves, as indicated in Figure 4, show how well the values of the power compare with the published data.

As was to be expected, the power absorbed by water or other non-inoculated culture media, in the absence of air flow, is proportional to the fifth power of the turbine diameter and to the third power of the agitator speed. No substantial differences between the power absorbed by water and the culture medium have been noted except for the case of foam formation where the apparent volume of the fermentation broth was increasing and there was a resultant reduction in the mechanical power absorbed.

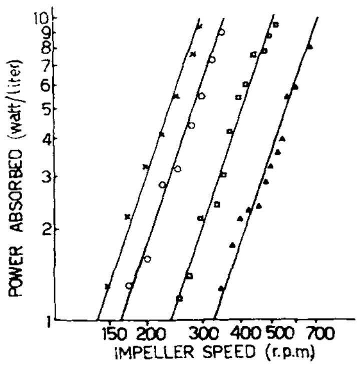

Fig. 4. The effect of the impeller speed and size on power absorbed for the agitation. Diameter of the impeller: △——△, 210 mm.; □——□, 250 mm.; O——O, 320 mm.; X——X, 360 mm.

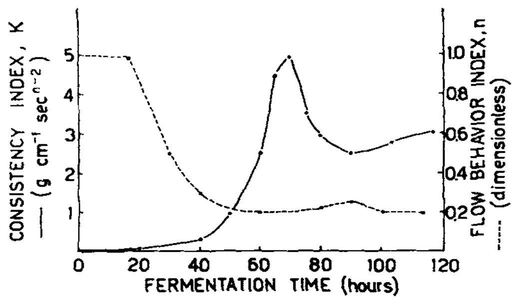

Fig. 5. Rheological properties of rifamycin fermentation broths.

However this case, owing to the continuous addition of antifoam, was rarely observed.

The analogous behavior between the broth containing mycelium and the broth alone can be attributed to the high turbulence (Reynolds number about 10^3) and to the viscosity of the fermentation broth, which never reached very high values (under 500 cp.).

And indeed, as can be seen from Figure 5, the rheological properties of the broths show first of Newtonian behavior and then, starting from the 30th hour of fermentation, a non-Newtonian behavior; at the same time the consistency index K rises from 1–3 cp. to about 400–500 cp.

2. The Effect of Power Absorbed on Rifamycin Yield with Impellers of Various Diameters

A series of rifamycin fermentations, with impellers of different diameters at constant power absorption, has been performed to point out the relationship existing between absorbed mechanical power, expressed in watts/liter of broth, and the production of antibiotic.

It is evident from Figure 6 that there is a range of absorption of power between 1.25 and 2.75 *W.*/l., within which the fermentation is influenced by the turbine's diameter, that is, the antibiotic production decreases as the turbine's diameter is increased.

For example, using a small turbine with a 210 mm. diameter, the maximum concentration of antibiotic is reached at a level of 2.5 *W.*/l.; using a turbine with a 360 mm. diameter, the antibiotic con-

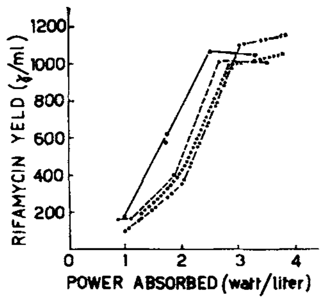

Fig. 6. The effect of power absorbed on rifamycin yield with various impellers. Diameter of the impeller: ——, 210 mm.; ----------, 250 mm.; ....., 320 mm.; ··— ··, 360 mm.

centration is about 40% less than that obtained with the small turbine.

Most likely, turbines with a smaller diameter, absorbed power being the same, bring about a higher degree of turbulence and a higher rate of oxygen transfer. Beyond the range of power absorption just examined, it is clear that the antibiotic production becomes independent of the turbine's diameter. And indeed, below 1.25 *W.*/l., the production of rifamycin is low and does not exceed 25% of the maximum production for all types of turbines. On the other hand, above 2.75 *W.*/l., with all turbines, the production of rifamycin is high and reaches its maximum value.

3. Relationship between Dissolved O_2 and Rifamycin Production

A better understanding of the observations made above can be inferred from the tests performed with the problem stated in terms of dissolved O_2.

Figure 7 shows the relationship existing between dissolved oxygen concentration and the concentration of rifamycin production at the levels of absorbed power below 1.25 and above 2.75 *W.*/l.; that is, those regions where the antibiotic production is independent of turbine size and of agitator speed. It is evident that the curves of dissolved oxygen, for the two different regions of power absorbed, differ greatly from one another in the fermentation area between the 60th hour and the harvest. In this zone, indeed, the oxygen demand is the highest and its concentration reaches values practically

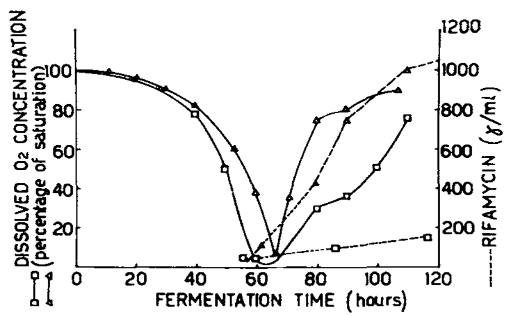

Fig. 7. Oxygen concentration and rifamycin production at different power absorptions. Impeller diameter: 250 mm.; power absorbed: □---□, 0.9 w./l.; Δ---Δ, 3.1 w./l.

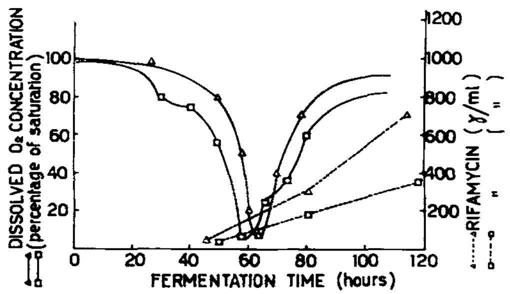

Fig. 8. Oxygen concentration and rifamycin production with different impellers. Power absorbed: 2 w./l.; diameter of the impeller: Δ---Δ, 210 mm.; □---□, 360 mm.

equal to zero. Probably the O_2 diffusion rate in the fluid could be the factor limiting the respiratory process, especially in the case of the fermentation carried out at a 0.9 *W*./l. power absorbed level.

Further investigations along this line were attempted to elucidate the relationship between diffused oxygen concentration and rifamycin production in fermentations where, keeping the power absorption level constant at 2 *W*./l., different diameter impellers were employed.

For our tests, two turbines, having, respectively, a 210 and a 360 mm. diameter, were chosen.

Figure 8 shows that with the smaller turbine the production of antibiotic was about 60% more than that of the fermentation using the larger turbine.

The most interesting aspect of these fermentations is that the oxygen concentration curve is very similar, for either test, to that of the previous diagram. The low production fermentation conducted with a 360 mm. turbine shows an oxygen curve very similar to that where the power absorbed was less than 1.5 *W.*/l. In the same way the high yield fermentation conducted with the 210 mm. turbine shows a curve of oxygen similar to that where the power absorbed is more than 2.75 *W.*/l.

It seems reasonable then to relate the rifamycin biosynthesis to the dissolved oxygen concentration, this latter being determined by the power absorbed and by the resulting diffusion speed. This seems to be of real importance in that zone of the fermentation cycle between the 50th hour and the harvest.

To verify this conjecture, a series of fermentations at various levels of power absorption and in different zones of the cycle have been conducted. The levels of power absorbed have been chosen in each case so as to be sure to obtain an oxygen curve corresponding both to a high and to a low dissolved oxygen concentration.

Particular attention has been paid to a zone of the fermentation cycle which, from previous observations, had been thought critical.

Table I indicates the typical periods in which the fermentation cycle has been subdivided, the levels of power absorbed by each of them and the final yields of rifamycin. For all fermentations, the same 250 mm. diameter turbine has been used.

It seems to be confirmed that in the rifamycin fermentation the critical zone of the cycle, as to the power absorbed, appears to be always between the 50th and the 80th hour. Keeping the power ab-

TABLE I
The Effect of Power Absorption Pattern on Rifamycin Production

Fermentation	Power absorbed, *W.*/l.			Final yield of rifamycin, γ/ml.
	0–50 hr.	50–80 hr.	80–120 hr.	
1	3.1	3.1	3.1	1.020
2	0.9	3.1	3.1	1.100
3	0.9	3.1	0.9	1.060
4	0.9	0.9	0.9	165
5	3.1	0.9	0.9	172
6	3.1	0.9	3.1	155

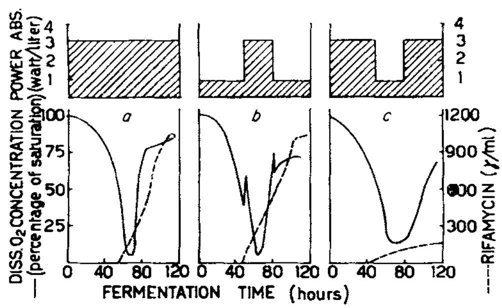

Fig. 9. The effect of power absorption on O_2 concentration pattern and its relation to rifamycin production. Diameter of the impeller: 250 mm. *a*. Fermentation No. 1, Table I; *b*. Fermentation No. 3, Table I; *c*. Fermentation No. 6, Table I.

sorbed at a high level (3.1 *W*./l.) during the mentioned period, the antibiotic production was at its maximum (fermentations No. 1, 2, and 3) independently of the mechanical power, absorbed before and after the critical period. Conversely, when this level of power during the same critical period was reduced to 0.9 *W*./l., the production of the antibiotic was low and, as before, it was independent of the power supplied before and after the critical period.

In Figure 9*a*, *b*, and *c* are diagramed the characteristic curves of the dissolved oxygen concentration for fermentations No. 1, 3, and 6, respectively (Table I).

The dissolved oxygen curves for the high yield fermentations (1 and 3) are much alike and they show, where oxygen demand and viscosity are higher, a fall in the oxygen concentration followed by a quick reestablishment of a probable optimum level. The curve representing fermentation No. 8 shows in this case, as in analogous cases in past observations, a behavior related to a low oxygen diffusion speed and, consequently, to a state of deficiency. This latter condition manifests itself by a curve that remains at very low levels of O_2 concentration for a period of time longer than in the previous cases.

After the previous observations it seemed appropriate to investigate more closely the behavior shown by the fermentations in Figure 7. In fact, we note that

(*a*) The oxygen curve, corresponding to fermentations carried out with an absorbed power for agitation equal to 0.9 *W*./l., has a lower level and a more flat course than the curve corresponding to fermentations where the absorbed agitation power was 3.1 *W.*/l.

(*b*) The same curve corresponds to a low rifamycin yield.

At this point we had to ascertain whether the lower O_2 level did not bring about an inadequate fulfilment of the O_2 demand for high rifamycin production.

In order to do it fermentations were performed with levels of absorbed power of 3.1 *W.*/l. and 0.9 *W.*/l. giving, respectively, a high yield (1100 γ/ml.) and a low yield (200 γ/ml.).

At different times the following were measured:

(*a*) The oxygen uptake (with a conventional manometric technique) representing the maximum O_2 demand of the microorganism in optimum conditions of high dilution. The sample for the Warburg apparatus was diluted from 10 to 20 times with filtered broth, depending on the respiratory activity of the microorganism.

(*b*) The actual oxygen uptake in the fermenter with a polarographic measurement of the O_2 percentage in the consumed air.

It was observed that

(*a*) In both cases, as was to be expected, the O_2 uptake is smaller than the O_2 demand.

(*b*) In conditions of greater agitation (absorbed mechanical power equal to 3.1 *W.*/l.) the O_2 uptake and the O_2 demand practically coincide till the 70th hr. (Fig. 10*a*), thus showing the optimum conditions for oxygenation. From the 70th to the 90th hr. the O_2 uptake falls faster than the O_2 demand; this is due most likely either to a greater resistance to the transfer of O_2 from the solution to the cells or to an incomplete degree of homogeneity.

Nevertheless, the decrease in the O_2 uptake is followed by a parallel and rapid reduction in the O_2 demand.

This course, coinciding up to the 70th hr. and then parallel up to the 90th hr., leads us to believe that in the case of fermentations performed under these conditions, the oxygen supply is substantially adequate for all phases of the microorganism's growth and for rifamycin production.

(*c*) In conditions of reduced agitation (absorbed mechanical power equal to 0.9 *W.*/l.), the curve representing the O_2 uptake shifts away from the curve of the O_2 demand much sooner than in the previous case (Fig. 10*b*). Moreover, the curve representing the O_2 demand slowly rises to a maximum value corresponding to that ob-

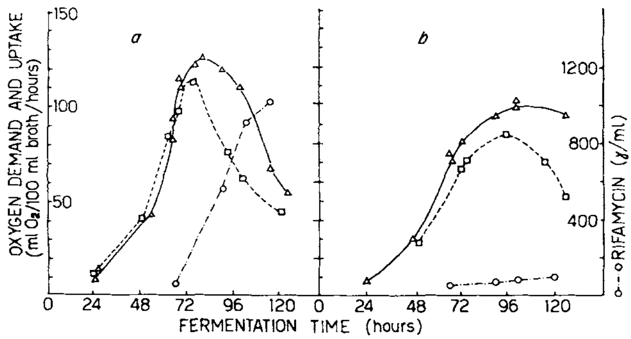

Fig. 10. Oxygen demand, oxygen uptake, and rifamycin production. Diameter of the impeller: 250 mm. *a.* power absorbed: 3.1 w./l.; *b.* power absorbed: 0.9 w./l. △——△, oxygen demand; □‐‐‐□, oxygen uptake.

served in the case of Figure 10*a*, thus showing a reduced metabolic activity and the initial phase of a delay in production to a later period. Meanwhile, the O_2 uptake shows a much lower value and a relatively rapid fall not parallel to that of the O_2 demand, which remains high until the 120th hr. of fermentation.

Accordingly it is interesting to note that, in high yield fermentations, the phase of maximum demand (corresponding from the morphological point of view to a massive, widespread fragmenting process of the hyphae of the microorganism) can be of a very limited length (about 20 hr.) and, consequently, the success of the process appears to be related to an efficient and sufficient aeration of this "critical" phase of the fermentation.

This is corroborated by the experiments reported in Figure 7 and in Table I.

CONCLUSIONS

Our observations can be summarized in the following points:

(*1*) For values of absorbed agitation power between 1.25 and 2.75 *W.*/l., the production of rifamycin decreases as the impeller diameter increases.

(*2*) Above 2.75 *W.*/l. and below 1.25 *W.*/l. the production is independent of the impeller diameter.

(*3*) In fermentations carried out at a level of 3.1 *W.*/l., the dissolved oxygen concentration drops below 40% of saturation for a period of about 15 hr., whereas in fermentations performed at 0.9 *W.*/l. such a period exceeds 40 hr. The production is 1100 and 190 γ/ml., respectively.

(*4*) The level of absorbed power being the same, the dissolved O_2 concentration goes below 40% of saturation for 10–15 hr. for propellers of smaller diameter, and for 30–40 hr., for propellers of larger diameter.

(*5*) It was found that, by keeping the power absorbed for the agitation at a high level (3.1 *W.*/l.) during a "critical period" between about the 50th and the 80th hr., the resulting antibiotic's production is high, independently of the mechanical power absorbed before and after this period.

(*6*) The agreement between the O_2 uptake and the O_2 demand is significantly better in fermentations performed at 3.1 *W.*/l. It appears reasonable to ascribe the higher yield of rifamycin to the fulfilment of the O_2 demand in the critical phase.

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