THE GAS REQUIREMENTS OF MOLDS. II. THE OXYGEN REQUIREMENTS OF *PENICILLIUM ROQUEFORTI* (THREE STRAINS ORIGINALLY ISOLATED FROM BLUE VEINED CHEESE) IN THE PRESENCE OF NITROGEN AS DILUENT AND THE ABSENCE OF CARBON DIOXIDE

N. S. GOLDING

Division of Dairy Husbandry, Agricultural Experiment Station, State College of Washington, Pullman, Washington

INTRODUCTION

The object of this study was to determine to what extent the O_2 of the air must be diluted with N_2 to show the inhibition of growth of strains of *Penicillium roqueforti.*

The previous study (4) has shown the marked difference in the growth of strains of *P. roqueforti* in an atmosphere diluted with N_2 as compared with CO_2. The study was continued with the object of determining the O_2 requirements of strains of *P. roqueforti* in CO_2-free air, diluted with N_2, over a wide range of temperature. The CO_2 in the air and that produced by the mold during growth were absorbed by NaOH solutions, to produce as far as possible the absence of CO_2 during growth.

LITERATURE

Since writing the previous paper (4) of this series, little work has been published on the O_2 requirements of any of the molds in the absence of CO_2. Certain new work refers to the need for O_2 in gluconic acid production by *Aspergillus niger* (6), but since, in the method described, the acid formed is neutralized by precipitated chalk ($CaCO_3$) it is questionable whether the presence of CO_2 is not the more important factor, which necessitates a frequent change of air during the fermentation.

CULTURES

Three cultures of blue mold, strains of *P. roqueforti,* from the previous study (4) were used, namely:

Culture 32. Isolated from a Wensleydale cheese made at the University of British Columbia.

Culture 33. Similar origin.

Culture 37. Isolated from a Wensleydale cheese made by Rowntree, York.

Received for publication February 12, 1940.

Published as Scientific Paper No. 454, College of Agriculture and Experiment Station, State College of Washington.

Studies of their cultural characteristics and morphology have been previously reported (2, 3).

Purification. Before commencing the study the cultures were repurified. The usual poured-plate technique was used. The medium employed was very clear whey gelatin, which, with the assistance of a low power binocular microscope, enabled the marking of the colonies soon after germination. When the marked colonies had grown somewhat, they were transferred to agar slants. This was repeated four times. While it was not possible to select individual spores, it is reasonable to conclude that each culture was obtained from the growth of one or more spores of the same origin.

MATERIALS AND METHODS

Medium. From the results in the previous study it was decided to use Difco malt agar. Thus, to prevent changing the medium during the experiment, 17 lbs. of dehydrated Difco malt agar was purchased and stored at approximately 40° F.

Slants. Agar slants of the above medium were used for carrying the cultures and also for making the water dispersion of the mold culture to inoculate the plates. When required for water dispersion, the inoculated agar slants were grown for 11 days at from 65° F. to 70° F.

Preparation of plates. As in the previous work (4), 25 ml. of the above medium were used for each plate. After pouring, the plates were put on a cold slab to solidify, which prevented condensation on the lids.

Inoculation of plates. One ml. of sterile water was added to the 11-day-old slant culture with a sterile pipette, and after mixing it was returned to a 100 ml. water blank and shaken well. A sterile "L"-shaped platinum wire was dipped into the water dispersion of mold spores and used to inoculate the center of the plate. Thus the colony started from a small hole of fairly uniform size made by the platinum "L." Examination under the binocular microscope showed that growth started from an area of 2 mm. or less. The same wire was used in all cases. For each growth curve determination, 5 plates for each culture at each temperature of incubation were inoculated.

Incubation. The same battery of incubators was used as in the previous study (4). Seven of these compartments were set to range in temperature at about equal intervals for a total variation of from 46° F. to 90° F. A thermograph or maximum and minimum thermometer was kept in each compartment to determine fluctuations in temperature. Also, 2 thermometers for each compartment were sealed with wax in small bottles containing water and read and recorded twice daily. The average of the readings of the latter was considered as the temperature of the incubator for the period of incubation. The slight variations in temperature from day to day seldom exceeded 1° F. In the later experiments Weston metallic thermometers were fitted into the desiccators and gave the temperature directly in contact with the plates.

Nitrogen. In this study, N_2 was the only gas used to dilute the air. The N_2 was used from the same commercial cylinder of gas.

Gas chamber. As in the previous study (4) 250-mm. Pyrex vacuum desiccators were used as the gas chambers for growing the molds in all cases. The maximum capacity of these desiccators is 22 Petri dishes each, when using only the space above the desiccator plate. One desiccator was put in each incubator compartment and connected with 2 glass tubes. One tube was connected to a common gas line to a mercury manometer, a suction pump, and the nitrogen cylinder. The other gas tube was the air intake for each desiccator, which was connected to 4 gas bottles (specially-fitted quart milk bottles). The gas bottles, which were held at the same temperature as the desiccator, were in the following order: First, a bottle of a 10 per cent solution of NaOH to remove CO_2 from the air; second, a bottle of dilute solution of H_2SO_4 to prevent any NaOH being carried over into the last 2 bottles, which contained a saturated solution of $(NH_4)_2SO_4$ and its crystalline salt; the last bottle was connected to the desiccator. Thus the air drawn into the desiccator passed through NaOH to remove CO_2, then through H_2SO_4 and finally through a saturated solution of $(NH_4)_2SO_4$ to humidify the air to about 80 per cent and prevent the desiccation of the medium on the plates.

Experimentally, it was found that over a period of 7 days (the 10 per cent added N_2 growth curve) a plate lost not more than 1.0 gram at 87° F. or about 0.5 gram at 60° F. To remove CO_2 from the air in the desiccator, a porridge dish containing 5 per cent NaOH was held in the space below the desiccator plate.

During the growth period there was no need to move the desiccator from the incubator for changing the gas supply. Thus, variations in changes in temperature were avoided. Furthermore, the desiccator and gas wash bottles were held in their respective incubators before the plates were added, thus shortening the time for adjusting the temperature of the inoculated plates.

Adding and changing gases. The desiccators, all having been filled with the required inoculated plates, were simultaneously evacuated to reduce the content of the air to the required fraction. The reduced pressure was measured with the manometer. The N_2 was then added to the desiccators until atmospheric pressure was reached.

Example: Required: a mixture of 30 per cent of air and 70 per cent N_2. Barometer pressure 700 mm. The desiccators were all simultaneously evacuated to a column of mercury of $\frac{700 \times 70}{100} = 490$ mm. N_2 was then added to atmospheric pressure.

A daily change of gas was made simultaneously in all desiccators by bubbling air briskly through the solution in the 4 gas bottles and on through

each desiccator for 10 minutes. Thus, natural air free from CO_2 and at the specified humidity was obtained around all the plates in the desiccators. To change the gas over the plates, the desiccators were evacuated to 500 mm. back pressure and returned to atmospheric pressure via the gas bottles. This was repeated in all 3 times. When the molds were required to grow in a gas supply other than air free from CO_2, the desiccators were simultaneously evacuated to the required pressure (see above example) and then filled to atmospheric pressure. Both outlet and inlet to the desiccators were then closed; thus, only very slight deviation from atmospheric pressure occurred. The daily gas change took from 40 to 60 minutes. Being repeated 7 times for each growth curve of 168 hours, an unavoidable error of 4 to 5 hours growing in air is introduced in each growth curve.

Growth period. After inoculation, which required less than one hour, the plates were inverted and put in their respective desiccators and incubated for a period of 7 days.

Measurement of colony. Wherever possible, the growth of the colony of mold was expressed in millimeters representing the average diameter of 5 colonies. (There were very few exceptions where the organisms either failed to grow or became contaminated, in which case the average diameter was obtained from less than 5 colonies.)

Expression of growth. From the above measurements, curves have been drawn for each change in gas supply, using millimeters growth as the vertical axis and temperature as the horizontal axis. Such curves have the advantage of permitting:

1. The making of a control curve for all temperatures of growth. Thus, controls do not have to be run concurrently with each curve made under changed gas supply.

2. The interpolation of the average size of colony for any temperature over the range of growth for the culture.

3. By interpolation the plotting of a growth curve for any temperature, having growth as the vertical axis and concentration of any gas at the horizontal axis.

4. The capacity of the culture of mold to grow under a definite condition, to be expressed by the area enclosed by the curve. (The areas were expressed in the same units.)

5. The expression of growth in air, less CO_2 on the basis of 100 for all temperatures. Thus a comparison can be made with the growth of the same culture grown under any other gas supply at the same temperature, and in the same medium.

Example: Culture 32 D grown at 70° F. showed from the growth curve by interpolation a diameter of 55 mm. in air less CO_2. Culture 32 D grown at 70° F. showed from the growth curve by interpolation a diameter of 48 mm. in air less CO_2 20 per cent, added N_2 80 per cent.

Thus, as $55 : 48 = 100 : x$

$$x = \frac{48 \times 100}{55} = 87.3$$

NOTE: The method permits of seeing at once the percentage of variation on a common basis for all cultures and for any temperature of growth. Comparisons with many other organisms, which are adapted to the technique, can later be made.

Also, insignificant variations (*i.e.*, not exceeding 10 per cent from the control) are immediately apparent.

PRELIMINARY EXPERIMENT

An experiment was conducted according to the method already described to develop 3 control curves, namely:

1. Air less CO_2 (Approximately 21 per cent O_2).
2. Air with no CO_2 removed (Approximately 21 per cent O_2).
3. Air less CO_2 evacuated to 90 per cent vacuum to determine whether a high vacuum would effect the mold growth (Approximately 21 per cent O_2).

The curves are given in figures 1, 2, and 3 for the cultures 32 D, 33 D, and 37 D respectively; also in table 1. The curves for each culture show that there is not a significant difference of over 10 per cent except for curve 3 which is slightly irregular at high temperatures. The curves 1 (air less

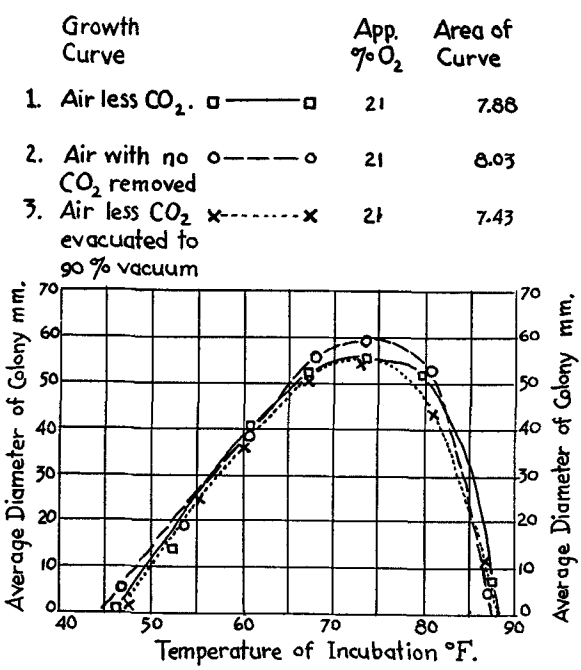

FIG. 1. Control curves to determine the significance of different techniques. Culture 32 D.

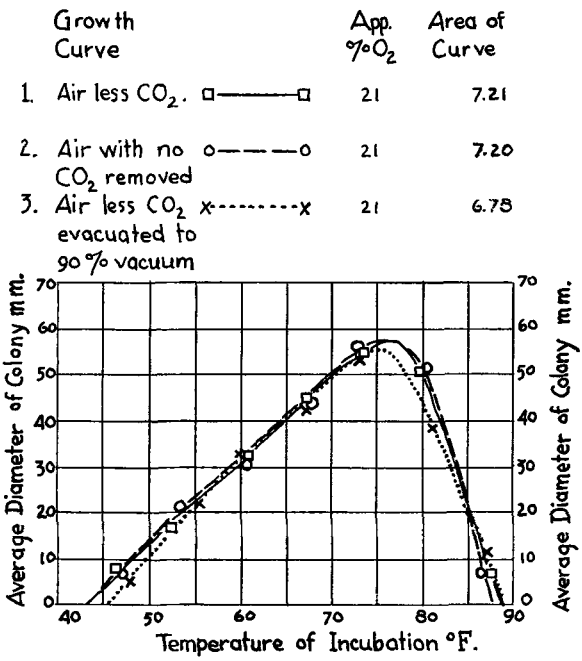

FIG. 2. Control curves to determine the significance of different techniques. Culture 33 D.

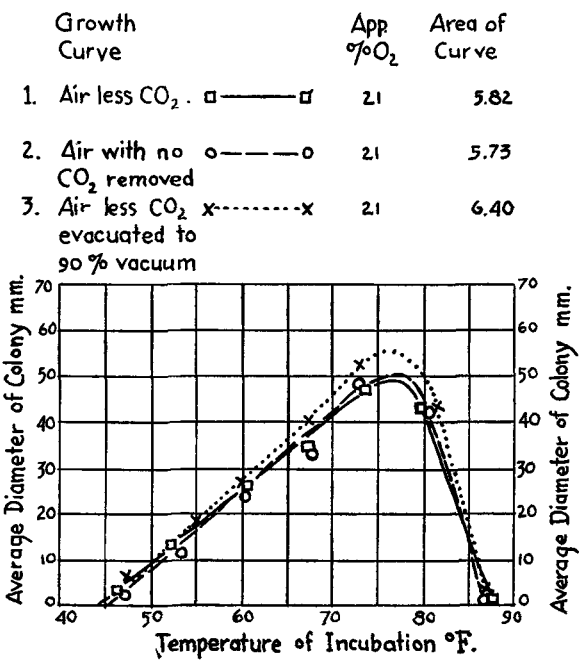

FIG. 3. Control curves to determine the significance of different techniques. Culture 37 D.

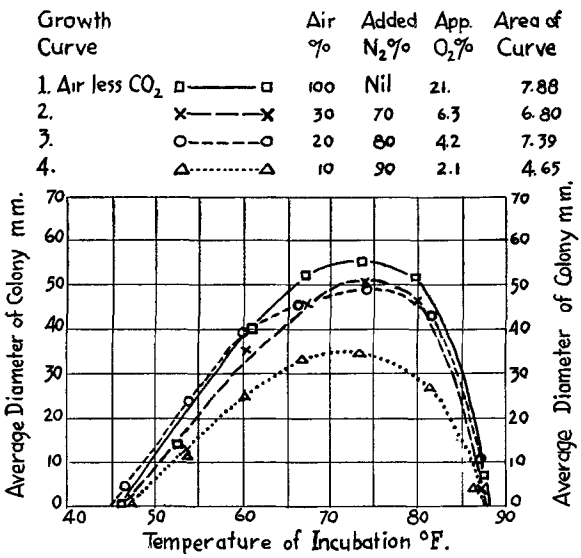

FIG. 4. The effect on the growth of *P. roqueforti* by the reduction of oxygen obtained by adding nitrogen, culture 32 D.

CO_2) and 2 (air with no CO_2 removed) for all 3 cultures nearly superimpose and would justify using either as control. In all subsequent experiments curve 1 (air less CO_2) was used as the control for comparison, with the other curves having a reduced O_2 supply in the absence of CO_2.

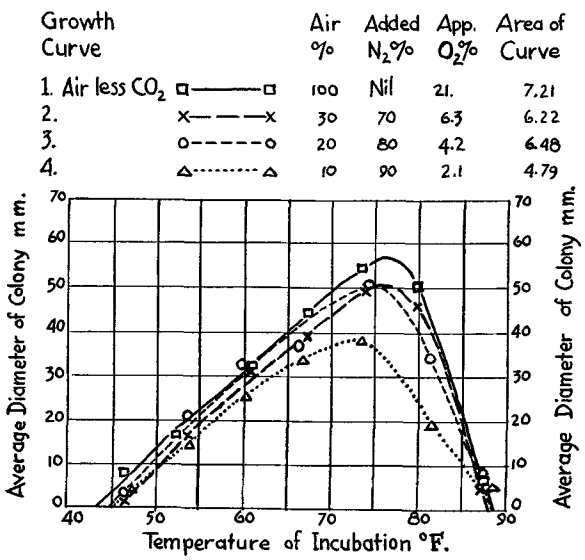

FIG. 5. The effect on the growth of *P. roqueforti* by the reduction of oxygen obtained by adding nitrogen. Culture 33 D.

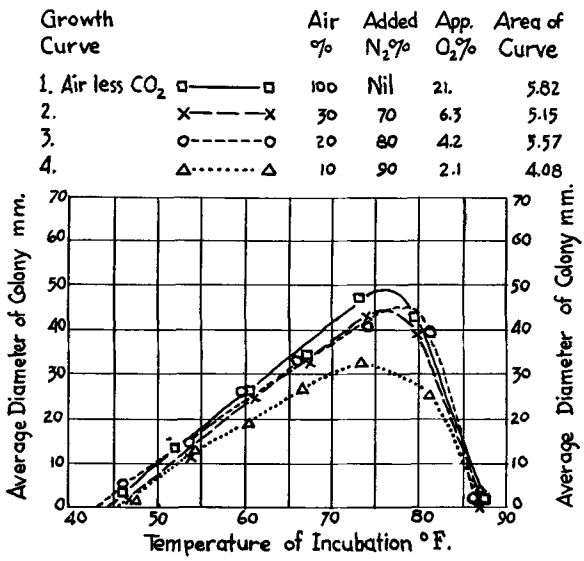

FIG. 6. The effect on the growth of *P. roqueforti* by the reduction of oxygen obtained by adding nitrogen, culture 37 D.

EXPERIMENTAL

The oxygen requirements in the presence of N_2 as diluent and the absence of CO_2

Figures 4, 5, and 6, growth curves for cultures 32 D, 33 D, and 37 D, respectively, show the seven-day growth curves when the air less CO_2 has

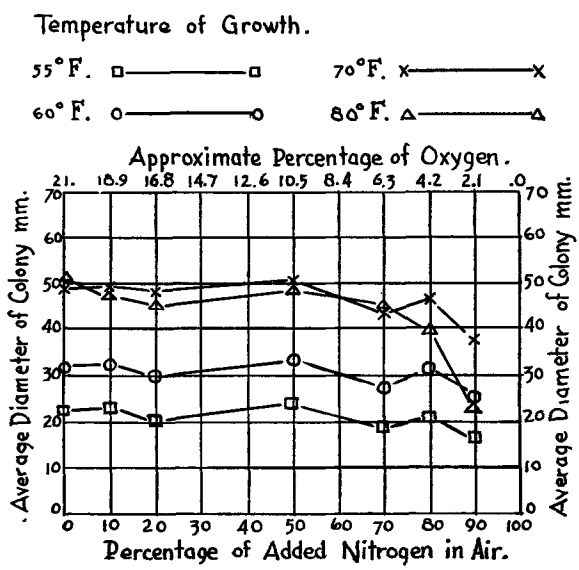

FIG. 7. The effect on the growth of *P. roqueforti* by the reduction of oxygen obtained by adding nitrogen. Culture 33 D.

TABLE 1

Acceleration or reduction in growth of cultures of P. roqueforti resulting from a change of gas supply. Expressed on the basis of growth in air less CO_2 at the same temperature as 100. Seven days' growth

Curve	Gas supply by volume			Culture	Temperature				
	Air % CO_2	Added % N_2	Approximate % O_2		55° F.	60° F.	70° F.	80° F.	Area
1	100 less CO_2	Nil	21	32 D	100	100	100	100	100
				33 D	100	100	100	100	100
				37 D	100	100	100	100	100
2	100	Nil	21	32 D	106	97	105	102	102
				33 D	102	103	100	100	100
				37 D	91	100	102	102	98
3	*100 less CO_2	Nil	21	32 D	96	92	100	90	94
				33 D	96	103	100	86	94
				37 D	103	108	112	117	110

* Evacuated to the same pressure as for 90% N_2.

TABLE 2

Acceleration or reduction in growth of cultures of P. roqueforti resulting from a change of gas supply. Expressed on the basis of growth in air less CO_2 at the same temperature as 100. Seven days' growth

Curve	Gas supply by volume			Culture	Temperature				
	Air % (Less CO_2)	Added % N_2	Approximate % O_2		55° F.	60° F.	70° F.	80° F.	Area
1	100	Nil	21	32 D	100	100	100	100	100
				33 D	100	100	100	100	100
				37 D	100	100	100	100	100
	90	10	18.9	32 D	108	100	100	100	102
				33 D	104	110	102	98	98
				37 D	97	104	107	105	103
	80	20	16.8	32 D	110	103	104	100	103
				33 D	89	97	98	92	92
				37 D	94	100	100	93	97
	50	50	10.5	32 D	108	103	98	92	99
				33 D	109	110	104	102	105
				37 D	100	100	98	98	100
2	30	70	6.3	32 D	84	85	89	88	86
				33 D	84	90	90	90	86
				37 D	89	92	93	90	88
3	20	80	4.2	32 D	108	102	87	87	94
				33 D	96	103	96	80	90
				37 D	94	96	90	102	96
4	10	90	2.1	32 D	64	64	64	56	59
				33 D	76	84	76	46	66
				37 D	72	76	73	64	70

been diluted with 70, 80, and 90 per cent N_2 respectively, as compared with the control air less CO_2. The additional seven-day growth curves of the 3 cultures when the air less CO_2 has been diluted with 50, 20, and 10 per cent N_2 respectively are not given as they superimpose with the control. However, these results obtained from the curves by interpolation are shown in figure 7 for culture 33 D.

Figure 7, for culture 33 D, was plotted by interpolating the points from the seven-day growth curves for temperatures of 55, 60, 70, and 80° F. and show by another expression the effect of reduced oxygen supply by adding N_2 in the absence of CO_2 on growth.

Table 2 shows the acceleration or reduction in growth of the cultures on a percentage basis, resulting from a reduction in O_2 by the addition of N_2 to the gas supply.

Together, the growth curves, figures 4, 5, and 6, with growth plotted against concentration of gas, figure 7 and table 2 in which growth is expressed on a percentage basis show:

1. A very low O_2 concentration, in the order of less than 4.2 per cent, is required before growth can be significantly reduced.*

2. There is a definite trend for the reduction in growth—caused by low O_2, shown in curves 5 and 6—to be proportionately greater above the optimum temperature of growth than that below the optimum.

3. Where temperatures below 55° F. are used, the period of 7-day growth is not sufficient to obtain large enough colonies for a good comparison.

4. The 3 strains of mold used show little difference in their response to the inhibiting effect of very low concentrations of O_2. However, it is fairly definite that culture 33 D is most affected above the optimum temperature of growth, while it is probably least affected below optimum temperatures.

DISCUSSION

The data in this paper agree with the data presented in the previous paper (4) insofar as the work is comparable. The length of the growth period in the previous study (4) was longer at the low temperatures of growth and the methods of adding the gas and handling the controls were sufficiently different to account for the small variations recorded. The data in this paper which indicate that no appreciable effect in growth is shown until very low concentrations of O_2 are reached are in agreement with the findings of Brown (1) using *P. glaucum* and *Fusarium* sp.

The reduction in growth of *P. roqueforti*, which is recorded by Thom and Currie (5), cannot in the least be attributed to low concentrations of O_2 which they obtained by adding CO_2.

* It was observed that the appearance of the colonies was not in the least changed, except in size, between the range of 21 and 2.1 per cent of O_2 when N_2 was used as the diluent of the air.

It would seem that the reduction of growth brought about by lowering the O_2 supply in the presence of N_2 first occurred at the high temperatures of growth. The cause of this has not been determined. However, should this be a function of the absorption coefficient of the gas (O_2), the problem of whether it is associated with the medium or the moisture content in the mold itself will have to be determined. Whatever the cause, there is no question that this part of the investigation still presents a valuable and interesting physiological study.

CONCLUSION

1. Three strains of blue mold (*P. roqueforti*) from cheese have been grown at 7 different temperatures in atmospheres of from 21 per cent to 2.1 per cent O_2, obtained by adding N_2 to the air. The results are expressed graphically.

2. It was only with the greatest O_2 dilution (2.1 per cent O_2) that a significant reduction of growth was recorded. This ranged between 16 per cent and 54 per cent.

3. It would appear that there is a tendency for this same shortage of O_2 to reduce growth more at the higher temperatures.

REFERENCES

(1) BROWN, WILLIAM. On the germination and growth of fungi at various temperatures and in various concentrations of oxygen and of carbon dioxide. Ann. Bot., 36: 257–283. 1922.

(2) GOLDING, N. S. The mold associated with the ripening of blue veined cheese. Mycologia, 17: 19–32. 1925.

(3) GOLDING, N. S. Some factors affecting the growth of *Penicillium roqueforti* in cheese. Ph.D. thesis, Iowa State College, Ames, Iowa. 1929.

(4) GOLDING, N. S. The gas requirements of mold. I. A preliminary report on the gas requirements of *Penicillium roqueforti* (various strains of blue mold from cheese). J. DAIRY SC., 20: 319–343. 1937.

(5) THOM, CHARLES, AND CURRIE, JAMES N. The dominance of Roquefort mold in cheese. J. Biol. Chem., 15: 249–258. 1913.

(6) WELLS, P. A., MOYER, A. J., STUBBS, J. J., HERRICK, H. T., AND MAY, O. E. Gluconic acid production. Effect of pressure, air flow and agitation on gluconic acid production by submerged mold growths. Indust. Eng. Chem., 29: 6, 653–656. 1937.