SUBMERGED CULTURE FERMENTATION

Factors Affecting the Production of Ustilagic Acid by *Ustilago Zeae*

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A study has been made of optimum conditions for ustilagic acid production by *Ustilago zeae* in submerged cultures. Tests were run in specially constructed 5-liter fermentors and successfully scaled up to 200-gallon fermentors, using sulfite oxidation rates as the criterion for aeration and agitation. Methods of recovery are described for ustilagic acid and useful degradation products derived from it.

An insoluble crystalline product termed "ustilagic acid" is formed when certain strains of the corn smut fungus, *Ustilago zeae*, are fermented in submerged culture. In previous papers from this laboratory the characterization of this mixture of monoacidic D-glucolipides has been described (*8*, *9*, *11*, *12*). Initially investigated because of their antibiotic activity (*5*), these compounds are now considered to be of more interest as precursors in the synthesis of musks for use in the perfume industry (*10*). On degradation, two closely related hydroxyhexadecanoic acids, termed ustilic acids, are produced and equivalent amounts of the 3-hydroxy-L-hexanoic and 3-hydroxy-L-octanoic acids can be recovered separately. All these compounds are potentially valuable (*10*).

The present investigation was undertaken to determine the optimum conditions for the fermentation in stainless steel fermentors and to develop suitable methods for recovery of the desired products. A further aim of the experiments was the development of small fermentors of known characteristics which could be used for the rapid evaluation of other fermentation processes.

Fermentors

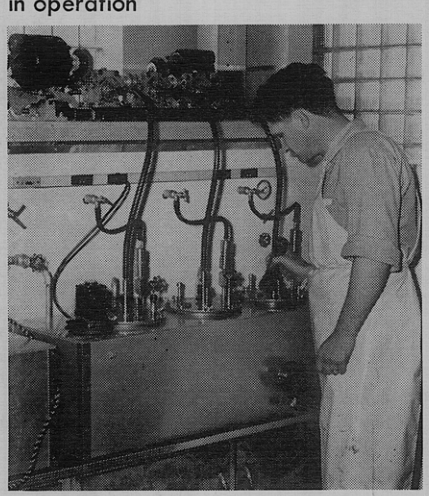
If a suitable basis for the scaling up of a fermentation can be found, the investigation of variables and determination of optimum conditions are most conveniently carried out in small fermentors. Preliminary experiments showed that the major factor in scaling up this fermentation was the degree of aeration required. Several methods, varying in degree of complexity, have been suggested for the scaling up of aeration and agitation (*1–3*, *6*). In the simplest case, where geometry of the fermentors is similar, measurements of the rate of sodium sulfite oxidation catalyzed by copper ion will provide an adequate basis for scaling up.

Five-liter fermentors made from stainless steel beakers were used in this investigation. Twelve of these were mounted in banks of three in controlled water baths (± 0.5° C.) with common agitator drives through flexible shafts and gear boxes. Figure 1 shows two such banks in operation. Speed of rotation was continuously variable from 250 to 800 r.p.m. Critical dimensions of these fermentors are given in Table I. In some of the earlier work a smaller agitator (2.75 inches in outside diameter, blade area 1.5 sq. inches) was used.

Sulfite oxidation tests (*3*) were run on these fermentors over the full range of agitator speeds and air flow rates to be used in later fermentations and small alterations were made in impellers and baffles to make their characteristics as nearly alike as possible. Final tests showed all to have oxygen uptake rates, using 5% sodium sulfite, within ±4% between 300 and 600 r.p.m. Appropriate small adjustments in air flow rate to individual fermentors can be made to remove even these small variations.

Existing 5-gallon and 200-gallon fermentors were also used, and sulfite oxidation tests were made over the normal range of operating conditions. The five-gallon fermentors were used only to prepare inoculum for the 200-gallon fermentations. As the 200-gallon fermentors showed low sulfite oxidation rates in comparison to the 5-liter size, the area of the baffles was increased by 280 square inches by extending them to the wall of the tank, which increased the aeration efficiency considerably. Dimensions before alteration are given in Table I. While the ratio of dimensions between small and large fermentors differs considerably in many cases, the ratio of height of liquid to diameter, which is most significant, is similar in the two cases. Impellers were positioned similarly in the two sizes of fermentors at about one third the depth of liquid from the bottom. In the 200-gallon fermentors air is sparged through the open end of the hollow agitator shaft, while in the small fermentors the sparger is a single hole in a pipe cap directly under the impeller and close to it. Baffles are of sufficient width in both cases so that the tanks may be properly classed as fully baffled.

Figure 1. Bank of 5-liter fermentors in operation



The results of sulfite oxidation tests on the 200-gallon fermentors are given in Table II. The superficial air velocities required for efficient aeration were very high because of inefficient baffle design, even after alteration. The level of aeration in 0.5-liter shake flasks, using 100 ml. of sulfite solution, is included for comparison with the fermentors.

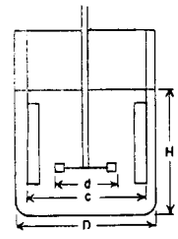
Analytical Methods

Analytical methods for ustilagic acid and its degradation products have been reported (*15*), but these were not suitable for routine controls. Methods were developed which gave results precise enough for the comparative determinations required and yet were simple enough for routine work.

Ustilagic acid is soluble in methanol and insoluble in water. As it contains 2 moles of glucose per mole, the standard anthrone method for carbohydrate (*4*,

Table I. Fermentor Dimensions

	200-U.S. Gallon Fermentors	5-Liter Fermentors
D, inches	36	7.1
d, inches	10	3.75
H, inches	27.2	4.6
A, sq. inches	27	5.0
V, imp. gal.	100	0.66
a, sq. inches	160	6.5
c, inches	22	5.5
d/D	0.28	0.53
c/D	0.61	0.78
H/D	0.75	0.65
A/V, sq. inch/gal.	0.27	7.6
a/V, sq. inch/gal.	1.6	9.8



A. Area of impeller blades (total)
V. Capacity at operating level
a. Area or baffles (submerged)

13), applied to a methanol extract of the washed fermentation solids, gives a convenient estimate. A suitable sample of fermentation broth is filtered, washed thoroughly with water, partially dried, and extracted with a volume of warm methanol equal to the initial volume of the sample. An aliquot of this is diluted 1 to 50 with water for the anthrone determination, using glucose as the standard for comparison.

This method, while convenient for routine determinations, is subject to error with certain strains of *Ustilago zeae* which produce anthrone-reactive materials, soluble in methanol, other than ustilagic acid. These have not been definitely characterized, but appear to be complexes of fatty acids (predominantly palmitic) and glucose, and are produced in lieu of ustilagic acid. A more specific analysis has been developed which is used periodically to check the simpler anthrone determination.

Ustilagic acid heated in methanol solution with an acid catalyst gives a quantitative yield of the methyl esters of the ustilic acids (*8*). These are soluble in chloroform but not in petroleum ether and can be recovered substantially free of impurities from the reaction mixture. In the method based on these properties, the methanol extract of the washed fermentation solids prepared as above is made 1.5N with hydrochloric acid and autoclaved at about 112° C. in a pressure bottle for 1 hour. An equal volume of chloroform is added and the methanol- and water-solubles are washed out with water. The chloroform layer is separated and evaporated to a small volume. Petroleum ether is added and the precipitate of methyl ustilates is collected in a weighed sintered-glass filter funnel, washed thoroughly with cold petroleum ether, dried, and weighed. This method gives reproducible results, but accuracy is increased by running a standard either of ustilagic acid or of methyl ustilates at the same time. Since the ustilic acids have terminal glycol groups, the purity of the methyl ustilates produced in this analysis or in production runs can be checked by colorimetric determination of the formaldehyde produced on periodate oxidation, using mannitol as the standard for comparison (*7*).

Fermentations

The medium used throughout was a simple one, previously developed for shake flask culture of the organism (*15*), containing per liter of medium: potassium dihydrogen phosphate, 1.0 gram; magnesium sulfate heptahydrate, 0.4 gram; ferrous sulfate heptahydrate, 0.03 gram; sugar beet molasses, 0.6 gram; urea, 1.2 grams; and calcium carbonate, 1.7 grams. Cerelose, used as the carbon source, was added in varying amounts from 50 to 180 grams per liter of medium. (Glucose monohydrate, manufactured by the Corn Products Refining Co., New York, N. Y., was used throughout. All sugar concentrations are calculated and reported in terms of glucose monohydrate.) For the purposes of inoculation *Ustilago zeae* was continuously subcultured in 500-ml. shake flasks containing 100 ml. of the basal medium and 7.5% glucose monohydrate. All fermentations were inoculated with 5% by volume of such subculture grown for 48 hours and pooled before addition to the fermentors. Inoculum for the 200-gallon fermentors was grown 48 hours in two 5-gallon fermentors using the same medium.

The factors considered in determining optimum conditions for pilot plant production of ustilagic acid were aeration rate and glucose concentration. Figures 2 to 4 show the effects of these variables on yield, efficiency, and rate of fermentation. Each point on these graphs shows the range of results obtained from a set of three to nine fermentations run in parallel from pooled inoculum. All fermentations were run at 30° C. with an initial glucose (monohydrate) concentration of 7.5% in aeration studies and an aeration level of 83 millimoles of oxygen per liter per hour in runs showing the effect of varying sugar concentration.

Three strains of the organism were used in the aeration experiments with little effect on the yield of ustilagic acid (Figure 2), although one strain showed substantially higher yields in one run under conditions of aeration equivalent to a sulfite oxidation rate of 82 millimoles of oxygen per liter per hour. Yields increased consistently up to the maximum aeration rate obtainable. Foaming could not be controlled at higher aeration levels.

The rate of fermentation increased with increasing aeration, as shown in Figure 3, but with much wider variations. One strain gave consistently lower rates and another was somewhat erratic. Although the general trends of fermentation rate and yield with increasing aeration are similar, a high fermentation rate in a given fermentation did not necessarily signify a high yield. A correlation exists only between fermentations run with the same strain of the organisms. The fermentation rate reported here is the maximum rate of sugar utilization found during the fermentation period, a nearly linear rate attained after a period of slowly increasing rate lasting from 6 to 24 hours, depending on the aeration level.

The yield and fermentation rate obtained in the 200-gallon pilot plant fermentors at a sulfite oxidation rate of 77

Table II. Rate of Oxygen Uptake by Sodium Sulfite Solutions at 25° C.

Fermentor Type	Agitator Speed, R.P.M.	V_s[a], Cm./Min.	Oxygen Uptake, Mmoles/Liter/Hour
Small agitator on 5-liter fermentor	550	3.80	32
	650	3.80	59
	750	3.80	80
Large agitator on 5-liter fermentor	300	3.80	55
	400	3.80	82
	600	3.80	129
	600	2.88	93
	600	5.76	162
5-Gallon fermentors	350	7.32	60
	400	7.32	83
200-Gallon fermentors, original design	300	26.2	55
	400	26.2	66
New design	300	26.2	60
	400	26.2	92
	400	17.3	77
0.5-Liter Erlenmeyer on Gump shaker	230	...	18

[a] Superficial air velocity.

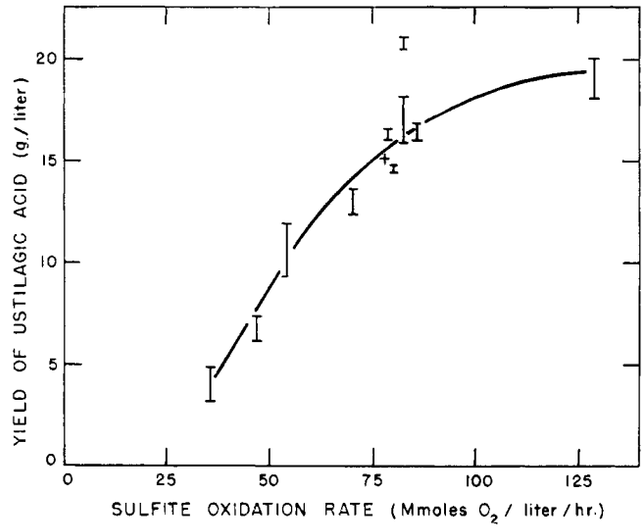


Figure 2. Yield of ustilagic acid in 5-liter fermentors

As a function of aeration rate as measured with 5% sodium sulfite solution under the same conditions of air flow and agitator speed. Fermentations at 30° C. with 7.5% glucose monohydrate. + 200-gallon fermentors

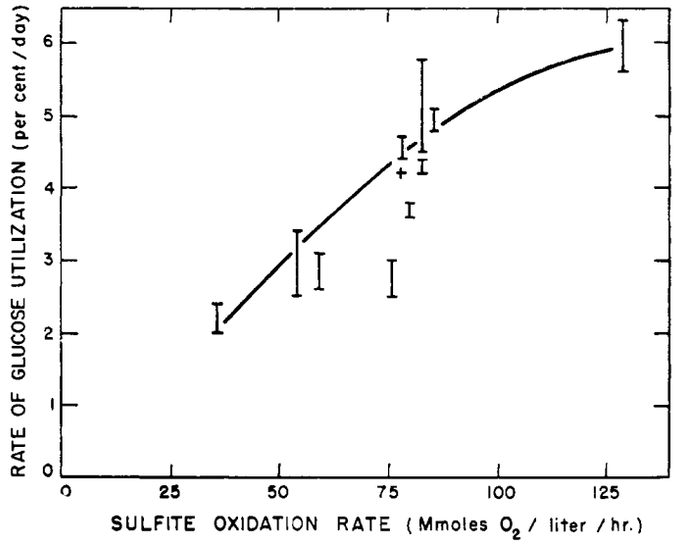


Figure 3. Rate of utilization of glucose in 5-liter fermentors

Measured at maximum value attained during fermentation. Fermentations at 30° C. with 7.5% glucose monohydrate
+ 200-gallon fermentors

millimoles of oxygen per liter per hour are also shown in Figures 2 and 3 by the symbol +. Although fermentations are somewhat slower in starting in these large fermentors, the eventual yield and the rate of fermentation during the linear period compare very closely with results at the same sulfite oxidation level in the small fermentors with the same strain of *Ustilago zeae*.

A definite optimum in the initial glucose concentration is shown in Figure 4. The maximum efficiency, calculated on the basis of the ratio of carbon recovered as ustilagic acid to carbon added as glucose, is shown to be about 32% at 10% glucose and an aeration level of 82 millimoles of oxygen per liter per hour. This is equivalent to a yield of 0.23 pound of ustilagic acid per pound of anhydrous glucose. Foaming interfered with runs in the small fermentors at high sugar concentrations at higher aeration levels, but a few abortive fermentations indicated that efficiency cannot be maintained with high aeration levels at an initial glucose concentration of 12.5%. The maximum rate of fermentation drops substantially at higher initial sugar concentrations, as shown in Figure 5. Experiments in which glucose was added intermittently during the course of the fermentation failed to maintain the initial high rate of utilization. Final yields were closely comparable to those obtained when all the sugar was added at the start, and the total time for the fermentation was significantly longer.

Optimum conditions for the ustilagic acid fermentation in the basic medium tested are obtained with an aeration rate, as measured with aqueous sodium sulfite, of over 80 millimoles of oxygen per liter per hour and an initial glucose concentration of about 100 grams per liter, as monohydrate. Under these conditions a yield better than 22 grams per liter of ustilagic acid is obtained in 40 to 50 hours. Conversion efficiency of carbohydrate to ustilagic acid averaged 33%, based on carbon content.

The fermentation was successfully scaled up, on the basis of sulfite oxidation rates, to 200-gallon pilot plant fermentors, but comparison with shake flask data indicates the caution necessary in the use of this method. Ustilagic acid is produced in good yield in shake flasks under conditions comparable to a sulfite oxidation rate of 18 millimoles of oxygen per liter per hour. In closed fermentors an apparent aeration level of at least four times this value is required for comparable yields. It is apparent, therefore, that the sulfite method for estimating aeration requirements is satisfactory only as a comparative method between fermentors of somewhat similar design, and that results cannot be construed as giving even an approximate value for the rate of supply of oxygen to an actual fermentation.

Recovery of Products

The fermentation product, ustilagic acid, has been shown to be a mixture of two complex organic compounds of similar properties (*9–11*). On degradation by refluxing with methanolic hydrochloric acid, methyl esters of four desirable acids are produced quantitatively (*8, 11, 12*). The closely related ustilic acids (15D,16-dihydroxyhexadecanoic acid and 2D,15D,16-trihydroxyhexadecanoic acid) are readily separated from the 3-hydroxy-L-hexanoic and 3-hydroxy-L-octanoic acids, as the methyl esters of the former are insoluble in petroleum ether.

Recovery of the ustilagic acid mixture as such depends on its solubility in methanol and insolubility in water. The extraction of the relatively dry fermentation solids with warm methanol sufficient to form a 5% solution of the ustilagic acid present and subsequent dilution of this methanol extract with eight to ten volumes of water will produce a flocculent, semicrystalline precipitate, free of impurities with the exception of some free fatty acids, which can be removed by extracting with ether or petroleum ether. This precipitate is difficult to recover either by filtration or centrifugation in its highly swollen state.

Since the degradation products of ustilagic acid are desired for most foreseeable uses (*10*), the preferred method of recovery leads directly to methyl esters of the ustilic acid and of the two 3-hydroxy acids. The methanol extract of the fermentation solids, as above, is acidified with hydrogen chloride (5%) or concentrated hydrochloric acid (to 1.5*N*) and refluxed at atmospheric pressure for 6 hours. Alternatively, this solution may be autoclaved at 112° C. for 1 hour with equivalent results. Some of the methanol is removed by distillation and the residue is diluted with water. The methyl esters of the acids are extracted quantitatively with a small volume of chloroform, in which they are very soluble, separated, and washed free of water-soluble impurities. The chloroform is reduced to a small volume and petroleum ether is added. The methyl esters of the ustilic acids are insoluble in this mixture in the cold and are recovered essentially pure, by filtration and washing with petroleum ether. The methyl esters of the 3-hydroxy acids are recovered by evaporating the petroleum ether from the filtrate and distilling the residue under reduced pressure.

Recoveries of the methyl ustilates by this method have varied from 70 to 98% of the theoretical on the basis of ustilagic acid originally present in the fermenta-

tion broth. The major loss is in the initial extraction with methanol, which may be incomplete if too much water is present. Some strains of *Ustilago zeae* have produced considerable quantities of free fatty acids and of other materials of solubility properties similar to ustilagic acid which interfere with its recovery by reducing its solubility in methanol.

The methyl ustilates may be hydrolyzed to the free acids before recovery from the methanolic solution by further refluxing with sodium hydroxide and reacidification. The free acids are insoluble in the mixture and can be recovered directly by filtration. The ustilic acids so produced are impure (70 to 85%) and yields, after recrystallization from alcohol, are lower than for the methyl ustilates. The free 3-hydroxy acids in this preparation are soluble and are best recovered by ether extraction of the filtrate. They are not readily separated except by conversion back to the methyl esters and distillation.

Discussion

The use of small laboratory fermentors in the investigation of fermentation variables is not new, but many of the designs have varied radically from the usual large fermentor in which fermentations will eventually be run. The fermentors described in this paper are similar to those designed by Bartholomew *et al.* (*1*) except that they are of stainless steel and somewhat simpler in design. The characteristics of these small fermentors are not identical with those of the 200-gallon fermentors used, but the more significant dimensions (ratio of depth to diameter, impeller position, type and position of sparger) are similar. In these fully baffled tanks the *Ustilago zeae* fermentation can be scaled up on the basis of a simple sulfite oxidation test, using water as the test medium.

The characteristics of a fermentation are significant in experiments designed to determine aeration requirements. The organism under study gives a broth similar in some respects to that from a yeast fermentation. The viscosity increases moderately during the fermentation, but considerably more than with yeast. There is little tendency for the cells to settle out or form clumps. The oxygen requirements are comparatively low, and the optimum oxygen uptake rate for respiration corresponds with that for maximum product formation, as previous work by Shu with closed shake flasks has shown (*14*). Shu also showed that the rate of ustilagic acid production is limited by the specific oxygen demand of the cells and that the organism is capable of utilizing oxygen at low tension in this medium, a combination of factors which is favorable to the use of the sulfite method of determining aeration conditions suitable for the fermentation. Despite all these favorable factors, sulfite tests on shake flasks give no indication of the aeration required in fermentors.

The large discrepancy between apparent aeration requirements for efficient fermentations between shake flasks (in which oxygen supply has been shown to be limiting) and the fermentors cannot be explained by data presented here. Although many factors may contribute to this difference, until they are investigated and the actual oxygen tension in the fermentation medium is measured, it must be assumed that oxygen supply is probably not limiting in the ustilagic acid fermentation run in fermentors of this type. The necessity for a degree of geometric similarity between small and large fermentors for successful scaling up of fermentations by applications of this indirect method is evident.

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Figure 4. Variation of efficiency of conversion of glucose to ustilagic acid with initial glucose monohydrate concentration in medium

Fermentations at 30° C. and aeration equivalent to sulfite oxidation rate of 83 millimoles of oxygen per liter per hour

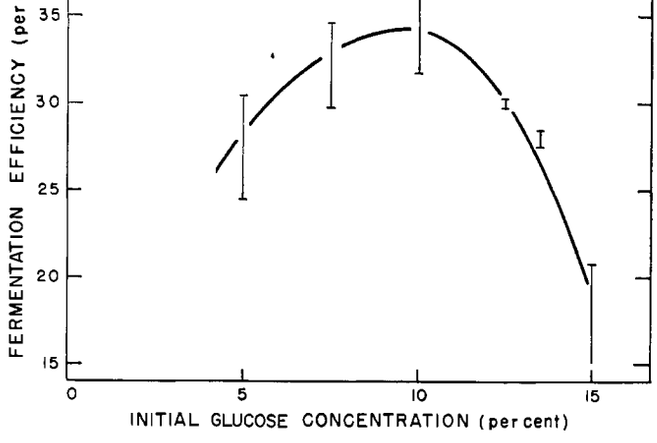

Figure 5. Variation of rate of glucose utilization with initial glucose concentration

Measured at maximum value reached during fermentation. Fermentations at 30° C. and aeration equivalent to sulfite oxidation rate of 83 millimoles of oxygen per liter per hour