Saccharification of Starchy Grain Mashes for the Alcoholic Fermentation Industry

Two strains of the mold *Aspergillus oryzae* have been found very satisfactory for producing amylase for use in saccharifying corn fermentation mashes. Best conditions for the production of highly active amylase preparations by growing the molds on wheat bran in rotating drums have been developed and are described. Data are presented for experimental fermentations in which the moldy bran was used for saccharifying the mashes; the effects of grinding, proportion of moldy bran, and temperature of saccharification were particularly investigated. Best results are obtained when the moldy bran is employed in the same manner as malt. On the average the moldy bran produces approximately 12 per cent higher alcohol yields than does a good dried barley malt. The possibilities of using the moldy bran to replace malt in the alcohol fermentation industry is discussed.

USE OF MOLD AMYLASE

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WHEN starchy substrates, such as the various grains or potatoes, are employed for the production of alcohol, the starch must first be converted to sugars before the yeast is introduced. This is usually done by adding malt (most commonly prepared by sprouting barley) which contains considerable quantities of the biological catalyst, or enzyme, of saccharification called "diastase" or "amylase." The authors prefer to use the term "amylase" since it avoids confusion that sometimes results from the fact that "diastase" is the French term for enzyme.

Many microorganisms produce amylase, and processes making use of microbial amylase for various purposes have been introduced into many countries. Certain molds have been found to be especially active in producing amylase. The literature on the use of microbial amylase in the fermentation industry has been reviewed by Smyth and Obold (*8*) and by Thaysen and Galloway (*11*). Amylase produced by molds has been used for centuries in the Orient, particularly in Japan, where *koji*, an amylolytic preparation obtained by growing suitable molds on steamed rice (*9*), is used instead of malt. It has been suggested that mold amylase might well be employed also in the fermentation industries of occidental countries. One development along this line is the Amylo process for the manufacture of alcohol, in which molds of the genera *Mucor* or *Rhizopus* are grown directly in the mash in order to convert the starch before yeasting. The papers of Galle (*4*), based on practical experience with the working of the Amylo process, and of Owen (*6*) most adequately describe the status of this method.

Takamine (*10*) patented the use of molds for the production of amylase preparations; one commercial product is marketed as "Taka-diastase." Use of mold preparations to replace malt in the fermentation industry was suggested by Takamine, and large-scale tests at the plant of Hiram Walker and Sons, Inc., in Canada in 1913 (*9*) proved entirely successful, yields of alcohol being better than with malt. However, a slight off-flavor or odor was produced in the alcohol, and since the flavor is of paramount importance in beverage alcohol, Takamine's preparation has not found favor in the alcohol industry. Now, however, with the increasing interest in power alcohol, it would seem that a procedure similar to Takamine's should hold much promise for production of industrial alcohol. Since the details of a workable process using mold amylase had not hitherto been well established, the purpose of the present investigation was to develop methods for culturing molds in order to produce an active amylolytic preparation, and to study the use of this material in saccharifying grain mashes for alcoholic fermentation.

Preliminary Studies

The saccharifying power of an enzymic material may be determined and expressed in degrees Lintner according to the Official Method of the American Society of Brewing Chemists (*1*) or by the method as modified by Anderson and Sallans (*2*). However, the Lintner numbers obtained for different materials, such as malt, soybean, different mold cultures, etc., have little meaning with regard to the relative merits of these different materials for saccharifying fermentation mashes. The usefulness of an amylolytic material for converting fermentation mashes can be determined accurately only by running fermentation tests. Hence, all results as given in this paper are based upon fermentation experiments.

Preliminary tests were made on amylase production by eight species of bacteria and twenty-one species or strains of molds. None of the bacteria gave very promising results. Of the molds tested, several strains of *Rhizopus* species and three strains belonging to the *Aspergillus flavus-oryzae* group were found to possess the highest amylolytic activity. For cultivation of the molds in flasks, several media were tried, including corn mash, moistened distiller's grains, and moistened wheat bran. The latter medium gave by far the best results and was therefore adopted for further work. Growth of the several *Rhizopus* species was in general less rapid and uniform than that of the *Aspergillus* cultures. Hence attention was concentrated on the Aspergilli. The three cultures selected, with their laboratory numbers, were as follows: culture 2, *Aspergillus oryzae*, secured from the American Type Culture Collection as No. 4814; culture 10, *A. flavus*, secured from Thom as No. 3538; culture 22, *A. oryzae* isolated from oat hulls by the author. The three selected cultures were continuously cultivated on agar slants and on moistened bran. Cultures 2 and 22 were plated to test for purity and no contamination was found. Individual colonies were selected for further cultivation.

The three mold cultures grew well on moistened bran. With cultures 2 and 10, only moderate sporulation occurred in 4 days, and incubation for at least a week was found necessary for abundant sporulation. Culture 22 produced a heavy crop of spores in 4 days. Likewise, the latter culture was observed to cause less lumping during growth. The optimum temperature for cultivation of the three mold cultures was found not to be very definite, but all grew best within the range 25° to 35° C.; the optimum was at about 30° C. Growth was somewhat limited at 38° C., and the cultures failed to grow at 42° C.

Since the growth of many microorganisms which might cause contamination of mold cultures is prevented by an acid reaction of the medium, use of dilute acid instead of water to moisten the bran in preparing the medium was investigated. Mold growth was satisfactory when hydrochloric acid up to 0.3 *N* was employed. Growth of culture 2 was a little more rapid when water was used, but sporulation was heavier in the acidified mashes. Little difference was observed in the growth or sporulation of culture 22 in either the mash prepared with water or with dilute acid.

Preliminary investigation of the amylolytic activity of the molds selected was made by means of the moldy brans resulting from the growth of the molds on moistened wheat bran in flasks. The moldy brans were used for saccharifying corn mash for fermentation tests; in the several series of fermentations different proportions of the moldy brans and various temperatures were used during the saccharification period. However, the results were exceedingly variable. This was believed to be due to the difference in the growth of the molds in the various flasks from day to day, and to lack of uniformity in the product which resulted from the excessive matting and lumping occurring during the growth. Hence, equipment was developed for cultivating the molds in larger quantities and in a more reproducible manner.

The apparatus developed (Figures 1 and 2) was simply a rotating drum through which a stream of sterile air could be passed for aeration of the growing mold. The drum used was a 5-gallon container (either a Pyrex bottle or an iron can) which was mounted on a system of rollers to serve as a support as well as a rotating device. The current of air could be passed into the drum by means of a glass or iron tube reaching through the stopper nearly to the bottom of the drum. The outlet for the air also passed through the stopper. The air was sterilized by bubbling through sulfuric acid and then humidified by passing it through sterile distilled water. The use of this equipment resulted in rapid mold growth with little matting and lumping; the moldy product was more uniform and possessed greater diastatic activity than that produced in flasks. All the data included in this paper were obtained with mold preparations produced in the rotating drums.

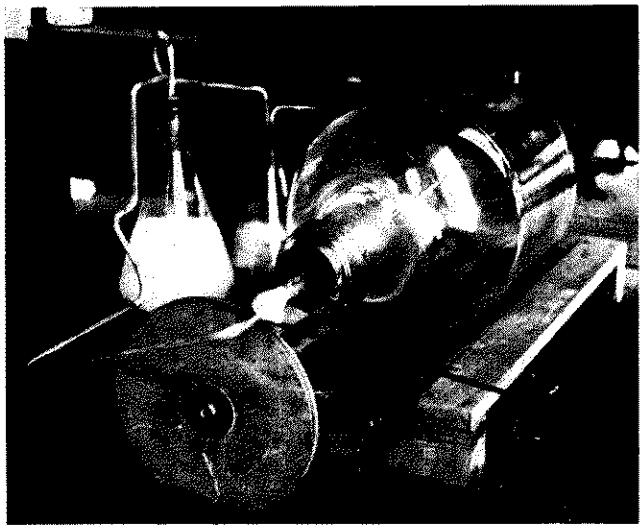

FIGURE 1. LABORATORY APPARATUS FOR GROWING MOLDS IN A ROTATING 5-GALLON PYREX GLASS BOTTLE (AERATION FROM THE COMPRESSED AIR SUPPLY)

Methods Adopted

As a result of the preliminary studies, the following methods were adopted for the study of mold amylase in saccharifying corn mash for alcoholic fermentation:

The stock mold cultures were grown on slants of sterile solid media in test tubes. Both wort-agar and glycerol–yeast extract–agar media were found suitable. For cultivating the molds in flasks, a bran mash was used. This was prepared by thoroughly mixing wheat bran with about an equal weight of distilled water and sterilizing for an hour at 20 pounds per square inch (1.4 kg. per sq. cm.) steam pressure. For best results it was found that the mash should contain from 50 to 70 per cent of water. About 30 grams of bran were used in a 500-ml. Erlenmeyer flask, or 50 grams in a 1000-ml. flask. Growth and sporulation were seriously retarded when larger quantities of bran were

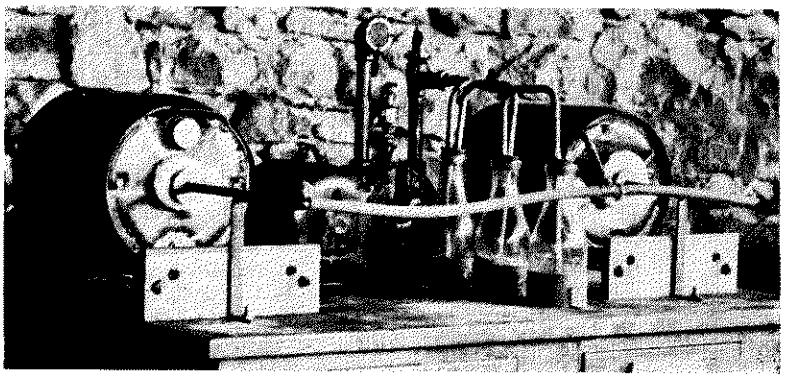

FIGURE 2. LABORATORY EQUIPMENT FOR GROWING MOLDS IN 5-GALLON IRON CANS UNDER FORCED AERATION AND WITH CONTINUOUS ROTATION

used, as a result of insufficient access of air to the mass. Rather heavy inoculations of the bran mash were made from well-sporulated slant cultures of the mold or, more conveniently, from well-sporulated cultures grown on the surface of sterile beer wort. The flasks were shaken every 4 to 8 hours to diminish matting and lumping of the contents as much as possible. Incubation was at 30° C.

MOLD GROWTH IN ROTATING DRUM. The drum is charged with 1000 to 1200 grams of wheat bran well mixed with the requisite amount of water to give a final proportion of 50 to 70 per cent moisture. The drum and mash are sterilized by heating in the autoclave for 2 hours at 20 pounds per square inch steam pressure. After being cooled to room temperature (about 25° C.), the mash is inoculated with 2 to 5 per cent of a well-sporulated bran mash culture of the mold grown in a flask. The contents of the drum are then mixed by rotation. A slow stream of air is passed into the drum, and during the germination period (from 12 to 16 hours under normal temperature conditions) the drum is rotated for not more than 15 to 20 minutes each 2 hours. The active growth of the mold becomes evident by a rise in temperature of the mass. After growth is well started, the rotation may be continuous or intermittent, but the speed of rotation should not surpass 1 r. p. m. Slow continuous rotation is preferable, and increased aeration with cool air saturated with moisture becomes necessary. The aeration is so regulated as to keep the temperature a little below 35° C. During the active growth period the bran assumes a white appearance due to mycelial development; sporulation of the mold changes the color to yellow-green. At the end of 40 to 54 hours the drum is stopped, and the moldy bran is spread out on papers and dried at room temperature. The dried material is then ground in a burr mill to a coarse powder and as such is employed to saccharify corn mashes for fermentation in much the same manner as malt is used.

FERMENTATION TESTS. Into each 1000-ml. Erlenmeyer flask were introduced 100 grams of corn meal, 1 gram of malt or moldy bran, and 500 ml. of water which had been heated to 80° C. The contents were mixed thoroughly by shaking and then allowed to stand for about 30 minutes. This "premalting" period was required to produce preliminary liquefaction of the raw starch and thus prevent the mash from becoming too thick and lumpy on subsequent cooking. After being cooked for 60 minutes at 20 pounds per square inch steam pressure, the mash was cooled to the conversion temperature and kept there by placing the flasks in a constant-temperature water bath. Weighed quantities of the amylolytic material were added, and the temperature was maintained for 60 minutes, the contents of the flasks being agitated frequently. After this period the mash was cooled to incubator temperature (30° C.) and inoculated with an active culture of yeast growing in beer wort or molasses medium, 50 ml. of inoculum being used per flask. After incubation for 3 days, the final volume of the fermented mash in each flask was measured, a 300-ml. aliquot distilled into a 100-ml. volumetric flask, and the alcohol content of the distillate determined by measuring the specific gravity (d_{25}^{25}) with a Chainomatic Westphal balance; the alcohol concentration was read from an appropriate table.

The yellow corn used throughout these experiments was of very poor quality (containing only about 50 per cent starch) as grown in Kansas during the drought year of 1936. The ground corn was analyzed according to the official, direct acid-hydrolysis method of the Association of Official Agricultural Chemists (*3*); the reducing substances formed were estimated by the method of Shaffer and Hartmann (*7*) and calculated as dextrose. The Shaffer-Hartmann reagents were standardized against a sample of pure dextrose.

The experimental results are expressed in the tables as per cent of the theoretical conversion of carbohydrate in the corn to alcohol according to the equation:

$$C_6H_{12}O_6 \longrightarrow 2CO_2 + 2C_2H_5OH \qquad (1)$$

The data given represent the average values for duplicate fermentations, and all yields were corrected for the amount of alcohol derived from the inoculum and amylolytic material. The method of calculation is illustrated by the following example: The final volume of beer in a fermentation flask was 600 ml., and the specific gravity of the distillate from 300 ml. of this beer was 0.9712, representing 18.05 grams of alcohol; the total alcohol content, then, was $18.05 \times 600/300 = 36.10$ grams. Analysis of control fermentations on the moldy bran employed in preparing the mash and on the beer wort used for growing the inoculum showed that 0.058 gram of alcohol was obtained per gram of moldy bran and 0.029 gram of alcohol per ml. of inoculum. Since 10 grams of moldy bran were used in preparing the mash and 50 ml. of inoculum in the inoculation, alcohol derived from the moldy bran and inoculum in the fermentation was $(10 \times 0.058) + (50 \times 0.029) = 2.03$ grams. Hence the alcohol produced from the corn was $36.10 - 2.03 = 34.07$ grams. From the theoretical equation, one gram of dextrose should give

$$2C_2H_5OH/C_6H_{12}O_6 = 92/180 = 0.5112 \text{ gram of alcohol}$$

The dextrose equivalent of the corn was 70.0 per cent; then from the 100 grams of corn used in the fermentation, the theoretical yield should have been $100 \times 0.70 \times 0.5112 = 35.78$ grams of alcohol. The actual yield of 34.07 grams therefore represents $100 \times 34.07/35.78 = 95.2$ per cent of the theoretical yield of alcohol.

Preparation of Moldy Bran

More than fifty batches of moldy bran have been prepared in the rotating drums. The first five runs served to determine the conditions of moisture content, sterilization, inoculation, rotation, and aeration required for the best growth of the molds. It was also determined during this preliminary period that the moldy bran could be air-dried without loss of amylolytic activity and could be stored for extended periods in the dry condition. The conditions found most suitable for preparation of moldy brans of highest amylolytic power are given above in the description of methods used and were adopted for all subsequent runs. For plant-scale use the moldy bran could be prepared in equipment similar to the pneumatic malting drums now in use.

Moldy bran preparations were made from each of the three selected cultures. The most rapid development of the molds began about 12 to 16 hours after inoculation, and mycelial growth was completed in about 40 to 54 hours. Abundant sporulation of culture 22 occurred within this period, but cultures 2 and 10 did not sporulate so heavily. Since the amylase is probably most abundant at the time of sporulation (*5*), it was desirable, when the moldy bran was to be dried, to remove it from the drums and spread it out for drying before extensive sporulation had taken place. It was then observed that during the drying period the sporulation ensued.

Fermentation of Corn Mash Saccharified with Moldy Bran

Several series of fermentations were run in flasks as outlined above for the purpose of testing the amylolytic activity of moldy bran preparations from the various runs and to determine the best method of using moldy bran for saccharification of the fermentation mashes to give maximum alcohol yields. The latter included investigations of the effect of drying the moldy bran and of grinding it, and determination of the optimum temperature for conversion of the mashes by the mold amylase and of the proportion of the moldy bran required to give maximum alcohol yields.

Each of the moldy bran preparations from the several runs was subjected to fermentation tests. The results of some typical fermentations are given in Table I. The mashes were saccharified according to customary malting practice for 60 minutes at 50–55° C. by means of air-dried and ground moldy bran, equivalent to approximately 10 per cent of the weight of corn in the mash. Tests 1 to 4 (Table I) were run at a different time from the others. The results show little difference in alcohol yields when moldy bran of either culture 2 or 22 was used for saccharifying the mashes. Culture 2 was apparently slightly superior. Rather uniform yields were obtained with the various preparations from different runs with the

same culture. Culture 10 yielded a preparation definitely inferior for saccharifying a fermentation mash. The better moldy brans gave greater alcohol yields than did malt controls, averaging about 12 per cent higher.

TABLE I. FERMENTATION TESTS WITH MOLDY BRANS

Test No.	Mold Culture No.	Drum Run No.	Alcohol Yield, % of Theory	Test No.	Mold Culture No.	Drum Run No.	Alcohol Yield, % of Theory
1	Malt	Control	80.0	8	2	19	93.9
2	2	5	78.0	9	22	5	92.4
3	2	9	83.5	10	22	16	91.6
4	2	10	87.6	11	22	19	91.3
5	2	11	90.0	12	10	8	68.7
6	2	13	97.4	13	Malt	Control	80.5
7	2	18	92.9				

A fermentation experiment in which varying proportions of both ground and unground moldy bran were employed in saccharifying the mashes showed that grinding the dried material increased the yields slightly at every proportion. For example, when 10 per cent moldy bran of the weight of corn was used, the alcohol yields were 76.4 and 78.8 per cent of theoretical for unground and ground material, respectively. Evidently the grinding process makes the enzyme more available for saccharifying the starch. The proportion of moldy bran required to give maximum alcohol yields was 8 to 10 per cent of the weight of corn used. The data of Table II confirm this proportion.

Table II shows the effect of the temperature of conversion and proportion of moldy bran required to produce maximum alcohol yields. The moldy brans used were dried and ground, and the mashes were saccharified at the indicated temperatures for 60 minutes. The results in *A* indicated that the highest conversion temperature employed (50–55° C.) is advantageous in the use of moldy bran from culture 2. In another experiment the results were reversed, although somewhat erratic; slightly higher alcohol yields were obtained with the conversion temperature of 40–45° C. There was little difference in alcohol yields throughout the range of temperatures used in the experiment with moldy bran from culture 22; a slight advantage appeared for the lowest temperature employed. These somewhat conflicting results lead to the conclusion that any temperature between 45° and 60° C. is suitable for the saccharification of corn mash before fermentation by moldy brans of cultures 2 or 22.

TABLE II. RESULTS OF VARIATION OF PROPORTION OF MOLDY BRAN AND OF SACCHARIFICATION TEMPERATURE ON ALCOHOL YIELDS

Saccharification Temp., ° C.	Moldy Bran, % of Corn (Dry Basis)	Alcohol Yield, % of Theory	Saccharification Temp., ° C.	Moldy Bran, % of Corn (Dry Basis)	Alcohol Yield, % of Theory
A. Culture 2, Run 13					
40–45	4.88	81.2	45–50	9.67	92.0
40–45	7.23	83.5	50–55	4.88	80.2
40–45	9.67	88.1	50–55	7.23	88.4
45–50	4.88	77.5	50–55	9.67	97.4
45–50	7.23	84.8			
B. Culture 22, Run 16					
45	10.56	93.9	50–55	10.56	91.6
60	10.56	91.3	50–55	7.92	92.0
			50–55	5.36	85.5

In order to test the suitability of moldy bran preparations for the conversion of starchy mashes on a larger scale, four culture tank fermentations were carried out at different times on about 85 gallons of mash in each case. The mashes were prepared and the fermentations were run as follows: Approximately 75 gallons of water were heated in the tank to about 80° C. Vigorous agitation was begun and continued throughout the mashing process. To the warm water were added 90 or 100 pounds of ground yellow corn with about 1 pound (1 per cent on the weight of corn) of malt or moldy bran, and the mash was "premalted" by maintaining the temperature at approximately 60° C. or 30 minutes. Water was then added to make the volume 85 gallons, the tank was closed, and the mash was cooked for 60 minutes at 15 pounds per square inch steam pressure. After being cooled to 55° C., the moldy bran was added to the cooked mash, and saccharification was carried out at 50–55° C. for one hour. The mash was then cooled to 30° C. and inoculated with an active yeast culture in molasses medium. Immediately after inoculation a sample of the mash was taken for the determination of total carbohydrate, reducing sugars, and acidity. The mash was agitated and vigorously aerated for 15 minutes in each hour for the first 6 hours after inoculation. Twelve hours after inoculation the mash was sampled and analyzed for alcohol content and acidity. Subsequently, sampling and analysis were repeated each 4 hours until the maximum alcohol concentration had been reached. Residual carbohydrates were then determined in a sample of the fermented mash or "beer."

In the two cases where sufficient of the ground moldy bran was employed for adequate conversion, the alcohol yields from the culture tank fermentations were as good as or better than those usually obtained with malt. Typical data are given for one culture tank fermentation:

Weight of corn used, 100 lb.
Total weight of starch in mash, 50.62 lb. (= 56.24 lb. dextrose)
Moldy bran used, 9.9 per cent of corn (dry basis)
Total carbohydrate (as dextrose) in mash, 56.5 lb. (7.96 gram per 100 ml.)
Residual carbohydrate (as dextrose) in beer, 5.66 lb. (0.80 gram per 100 ml.)
Carbohydrate utilized, 90.0 per cent
Alcohol yield, 90.1 per cent of theory (3.66 gram per 100 ml.)
Final acidity, 5.9 ml. of 0.10 *N* per 10 ml. of beer.

Conclusions

Fermentation tests in flasks have shown that moldy bran prepared with either of the two mold strains of *Aspergillus oryzae* (cultures 2 and 22) gives better results than malt in the saccharification of corn mash for fermentation; the alcohol yields are, on the average, about 12 per cent higher with the moldy bran than with malt. Moldy bran of culture 2 was found to produce slightly higher yields of alcohol than that of culture 22. However, the latter culture grows more rapidly with less tendency to lumping, produces spores more abundantly, and shows greater ability to overgrow contaminants, which would all be important factors when industrial use is contemplated. The moldy bran should be ground before being used in saccharifying mashes and may be employed either wet or dried. Air drying does not affect the amylolytic activity, nor does storing in the dry condition. A proportion of moldy bran of 8 to 10 per cent of the weight of corn in the mash seems to be necessary for maximum alcohol production, and saccharification can be carried out satisfactorily within the range 45–60° C.

Culture tank fermentations have shown that under favorable conditions alcohol yields of at least 90 per cent of the theory can be obtained by the large-scale use of moldy bran for saccharification of corn mash. This alcohol yield is rarely reached with malt, and it would seem that the preparation and use of moldy bran to replace malt in the production of industrial alcohol should hold much promise. The economic advantages of such a process are obvious. Not only can better alcohol yields be expected, but the high cost of malt can be largely eliminated. The raw materials for the preparation of mold amylase preparations, wheat bran, or other similar

bulky substance, are much cheaper than barley for the production of malt. Likewise, the duration of the process for the preparation is much shorter for moldy bran than for malt. For plant-scale use it should be possible to prepare the moldy bran without difficulty in equipment similar to the pneumatic malting drums now in common use. The product can then be ground wet and used like green malt, or dried and used like cured malt in the saccharification of the starchy mashes. The problems which are always encountered in going from the laboratory to the plant scale are now being investigated with regard to the production of the moldy bran. In the laboratory an investigation of the use of moldy bran for saccharification of starchy substrates other than corn is now in progress.

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Evaluation of Nitrocellulose Lacquer Solvents

Comparison by Means of a Constant Viscosity Procedure

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SEVERAL factors must be taken into consideration when a nitrocellulose solvent is evaluated. These factors vary in their relative importance according to the type of lacquer and the use to which it is applied. When contemplating the adoption of a certain solvent or solvent mixture for lacquer formulation it first becomes necessary to consider such items as the viscosity per unit of solids, hydrocarbon tolerance, cost, evaporation rate, flow-out characteristics, blush, and a number of others. However, the first three—viscosity, hydrocarbon tolerance, and cost—are of almost universal importance, regardless of the type of lacquer or its intended use. The object of this paper is to describe a method for nitrocellulose solvent evaluation by means of which a single value may be assigned to the combined viscosity characteristic and diluent acceptance of the solvent, and which, in turn, may be used to relate this combined solvency value to the cost of application.

Baker (*1*) in 1913 first advanced the use of viscosity of solutions as an indication of the solvent strength of the mixture for nitrocellulose; Sproxton (*7*) in 1920 used the tolerance of the solution for hydrocarbons for the same purpose. These two procedures have been employed extensively in the industry for comparing the ability of solvents to dissolve nitrocellulose, and no satisfactory method has been advanced to replace them up to the present time.

The viscosity and hydrocarbon dilution ratios of a lacquer solvent are commonly classed together under the heading of its "solvency characteristics", but only recently has any material appeared in the literature which attempts to interpret the two sets of data and correlate them into a more flexible and usable form. Doolittle (*2*) approached the problem by the utilization of phase diagrams which provide an evaluation of solvent strength by combining the two. It has been recognized for some time (*5*, *6*) that there are cases where dilution ratio and viscosity actually furnish conflicting indications. Of still greater importance in this connection is the fact that whether they are considered separately or together, they not only fail to supply a satisfactory basis for comparison, but in some cases they tend to exaggerate the differences which exist between two or more solvents or solvent combinations and to minimize them in others. Thus it is both difficult and confusing to attempt to assign a definite solvency value to one solvent as compared with another by considering separately viscosity, on the one hand, and hydrocarbon tolerance, on the other.

That there is a real need for a simple means of correlation of viscosity and dilution values becomes more evident when we consider the drawbacks associated with each of the two methods. The viscosity of a nitrocellulose solution alone in a pure solvent or solvent-coupler mixture does not serve as an adequate indication of solvent strength because costs depend largely upon the extent to which the solution may be diluted with cheaper hydrocarbon. On the other hand, dilution