THE EFFECT OF SUCCESSIVE GENERATIONS OF YEAST ON THE ALCOHOLIC FERMENTATION OF CIDER [1]

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INTRODUCTION

With the rapidly increasing demand for unfermented fruit juices during recent years much interest has been aroused in methods of manufacture. Although fruit juices are very attractive when first pressed they soon lose their attractiveness by yeast development, and consequently can not be handled or stored properly without being subjected to some process to check fermentation. Any treatment subsequent to pressing seems to injure more or less the delicate flavors and aromas of the juices. Filtering may improve the appearance but it does not improve the natural flavor. Chemical preservatives added in certain proportions may check fermentation but they often impart disagreeable flavors to the juices. Moreover, the use of such preservatives is of questionable propriety, and, as their presence is generally looked upon with suspicion by the public, their use is gradually decreasing. Pasteurization, although free from some of the objectionable features of the chemical preservatives, often gives a cooked taste and checks fermentation only so long as the treated juices are kept out of contact with the air. Refrigeration approaches perhaps most nearly the ideal of preserving the natural flavors of the juices, but it is an expensive process and unless the temperature is constantly kept at or near the freezing point of the juices fermentation starts readily and makes the product unfit for unfermented beverages. Since both pasteurization and refrigeration fail to produce unfermentable products, the objection that the juices so treated may be used for unlawful purposes remains unsolved. With the exception of the rather unsatisfactory use of chemical preservatives, there is yet no reliable method for the preparation of unfermentable fruit juices. In view of the rapidly growing demand for unfermented beverages it is evident that there is an urgent need for some practical method whereby clear, attractive, unfermentable fruit juices can be prepared with the least possible loss of natural flavors and aromas. There is still much to be learned about the fermenting organisms and their mineral food requirement. Careful research in this direction will undoubtedly bring to light much valuable information and may open the way to a successful solution of the unfermented-beverage problem. The work described in this paper is a preliminary study dealing with the application of some of the known basic factors in fermentation in an attempt to open the way to the development of some practical method of making unfermentable fruit juices without the use of chemical preservatives.

[1] Received for publication May 23, 1928; issued September, 1928. Published with the approval of the Director of the Washington Agricultural Experiment Station as Scientific Paper No. 148, College of Agriculture Experiment Station, State College of Washington.

SCOPE OF THE INVESTIGATION AND METHODS OF PROCEDURE

It has frequently been observed that bacterial cultures develop very poorly or refuse to grow at all in bouillon media that have once served for the growth of these same bacteria. It seems that the by-products accumulated by the first generation make the media unsuitable for the growth of successive generations. This observation is not limited to bacteria but applies equally well to yeast. When alcoholic fermentations are checked by rising temperatures, which are normal in active fermentation, it is more difficult thereafter to resume the fermentation in the normal way. The reasons generally ascribed for this behavior are that the increased temperature resulting from very active fermentation kills many of the active yeast cells and that the by-products of these dead cells retard or prevent the maximum development of the new generation. It is this assumption perhaps which induced Boulard[2] to study the phenomenon with the end in view of using it methodically in the control of alcoholic fermentations of wines. By growing successive generations of yeast in wines he succeeded in rendering them totally unfermentable long before all the fermentable sugars were used up. According to his expression these wines were "immunized" or "vaccinated" against yeast and also against spoilage bacteria. Although the causes of this so-called immunization or vaccination have not been studied and are as yet insufficiently understood, it is presumed that the accumulated by-products of preceding generations of yeast are toxic to the extent that further yeast or bacterial development is altered or even prevented. It is also thought that successive generations of yeast soon exhaust certain essential food elements so completely that the medium is no longer capable of supporting yeast and bacterial growth. These two assumptions constituted the main object of this study. Cider pressed from cull apples of mixed varieties was used in the experiments.

The toxicity studies were carried out by placing definite amounts of this cider in Erlenmeyer flasks, inoculating them with a heavy suspension of yeast culture, and incubating them at 25° C. Active fermentation was checked at will by heating the inoculated cider at 45° C. for one hour. As this treatment killed many of the active yeast cells, reinoculation following each heating was necessary. The process was repeated until the yeast refused to develop. Three or four inoculations were usually sufficient to accomplish this end.

Because of the well-known fact that cider is generally very low in nitrogen and phosphorus, both of which are believed to be indispensable for the growth and reproduction of yeast, the study of the possible depletion of certain food elements by yeast growth was limited to quantitative and qualitative determinations of these two elements. Determinations of alcohol and acetic acid were also made periodically but only for the purpose of following the course of their formation and of ascertaining how low their percentages could be kept during the process.

All the quantitative chemical determinations were made according to the official methods of the Association of Official Agricultural

[2] BOULARD. SUR UN PROCÉDÉ PERMETTANT D'ARRETER À VELONTÉ LES FERMENTATIONS A N'IMPORTE QUEL MOMENT. Compt. Rend. Acad. Agr. France 12: 615–620. 1926.

Chemists.[3] The qualitative tests for nitrites were made with Trommsdorf's reagent, those for nitrates with diphenylamine reagent, those for ammonia with Nessler's reagent, and those for orthophosphates with ammonium molybdate reagent followed by reduction with tin metal according to the method of Spurway.[4]

EXPERIMENTAL DATA

TOXICITY AND ALCOHOL PRODUCTION OF SUCCESSIVE GENERATIONS OF YEAST AND EFFECT ON NITROGEN AND PHOSPHORUS CONTENT OF CIDER

Three 2-liter Erlenmeyer flasks were filled with 1,500 c. c. of freshly pressed cider and two of them were heated at 45° C. for one hour and then cooled to 25°. Since heating caused considerable precipitation in the cider, the precipitate was first allowed to settle and was then separated by decantation. The supernatant liquid and sediment were analyzed separately for nitrogen and phosphorus. As the concentration of sediment naturally varied in different decantations, the results of the analysis of the various sediments could not be expected to agree very closely, but the analysis was made only to determine the approximate proportion of these elements in the two separated parts. When the analysis was completed the sediment was rejected but the flasks with the supernatant liquid of the heated cider and that with the unheated fresh cider were inoculated with 2 per cent of a heavy suspension of *Saccharomyces valesiacus* (Osterwalder) at its maximum activity in sterilized cider. The inoculum was thoroughly mixed with the cider in the flasks and allowed to develop at 25° until the fermentation became active as shown by the appearance of foam bubbles on the surface of the liquid. The three flasks were then heated at 45° for one hour, the heating causing once more considerable precipitation which was allowed to settle before being separated by decantation. The analyses for nitrogen and phosphorus of the supernatant liquid and sediment were made separately as before. Reinoculations with consequent heatings and analyses were repeated three times, resulting in progressively smaller amounts of precipitated material and a considerable decrease in yeast development each time. A fourth inoculation was made in which the amount of inoculum was twice that which was used previously. The cider at that time was already clear and remained so even after 24 hours' incubation at 25°. Neither was there the slightest indication of any yeast development observable by macroscopic means during that time. The results of the various analyses obtained up to this stage are reported in Table 1.

The data indicate that cider is very low in nitrogen and phosphorus, that a large part of these elements was easily precipitated by a temperature of 45° C., and that most of that remaining was used up by the first generation of yeast. However, the removal of the last traces of ammonia nitrogen and orthophosphates proved to be extremely difficult as indicated by the results of the qualitative analyses. A positive test for these substances was secured as long as the experiment was in progress, but the nitrogen was never found in the nitrite or nitrate forms in any of the experiments.

[3] ASSOCIATION OF OFFICIAL AGRICULTURAL CHEMISTS. OFFICIAL AND TENTATIVE METHODS OF ANALYSIS. COMPILED BY THE COMMITTEE ON EDITING METHODS OF ANALYSIS. REVISED TO JULY 1, 1924. Ed. 2, 535 p., illus. Washington, D. C. 1925.

[4] SPURWAY, C. H. TEST SOILS FOR WATER-SOLUBLE PHOSPHORUS. Mich. Agr. Expt. Sta. Quart. Bul. 9: 64-65. 1926.

TABLE 1.—*Analysis of cider and sediment in the first experiment at different stages of the treatment*

Treatment	Quantitative analysis in percentages						Qualitative analysis [a]			
	Supernatant liquid				Sediment		Supernatant liquid			
	C_2H_5OH by weight	Acidity in terms of CH_3COOH	Nitrogen	Phosphorus	Nitrogen	Phosphorus	Nitrites	Nitrates	Ammonia	Orthophosphates
Cider unheated before first inoculation:										
Fresh cider	0.00	0.40	0.0098	0.0028	-------	-------	−	−	+	+
First inoculation	-------	-------	.0	-------	0.035	-------	−	−	+	+
Second inoculation	-------	-------	.0	.0	.0056	0.0051	−	−	+	+
Third inoculation	.56	.44	.0	-------	.0002	-------	−	−	+	+
Cider heated before first inoculation:										
Fresh cider heated at 45° C. for 1 hour	.00	.39	.0056	.0013	.028	.0041	−	−	+	+
First inoculation	-------	-------	.0	-------	.039	-------	−	−	+	+
Second inoculation	-------	-------	.0	.0	.013	.0082	−	−	+	+
Third inoculation	.53	.44	.0	.0	.0024	.00	−	−	+	+

[a] +=positive test; −=negative test.

The effect of successive generations of yeast was shown by a cell development which was in inverse proportion to the number of generations. The third generation grew very poorly and the fourth failed to show any signs of growth for some time at least. By carefully checking the development of each generation as soon as a few foam bubbles appeared on the surface of the cider, it was possible to limit the formation of alcohol to as small amounts as 0.56 and 0.53 per cent. Although it is true that even the smaller amount is slightly in excess of the limits permitted by the law applying to unfermented beverages, probably by more carefully checking the development of the yeast this excess could be reduced sufficiently to comply with the requirements of the law.

The change in total acidity during the process was insignificant and the results in general were most gratifying in every respect up to this point.

At the end of 24 hours' incubation following the fourth inoculation plate counts for the number of living yeast cells in the treated ciders were made on plain agar supplemented with 10 per cent cider. This modified plain agar was used throughout this study because the yeast developed better on it than on the plain agar. The plate counts were repeated after 5 days' incubation and also after 10 days' incubation. The results together with those of the qualitative tests for ammonia nitrogen and orthophosphates are recorded in Table 2.

TABLE 2.—*The determination of active yeast cells, ammonia, and orthophosphates in cider at different stages of incubation after the fourth yeast inoculation in the first experiment*

Treatment	Living yeast cells per cubic centimeter [a]	Qualitative analysis [b]	
		Ammonia	Orthophosphates
24 hours after fourth inoculation	1,760,000	+	+
After 5 days' incubation at 25° C	950,000	+	+
After 10 days' incubation at 25° C	1,600,000	+	+

[a] Each figure in this column is the average of counts taken from 4 plates.
[b] The plus sign indicates positive tests.

In spite of the fact that there was no visible change in the cider during the first few days following the fourth inoculation the data show that the yeast cells seemed to remain alive. At least they did not die at any abnormal rate, nor did they develop to any noticeable extent. On the other hand, bacterial growth, probably resulting from air contamination, seemed to make some progress, for on the fifth day of incubation a very light film was plainly distinguishable on the surface of the cider. On the sixth day some activity, apparently caused by the production of gas, was noticeable, and on the seventh day it was quite evident that at least some of the yeast cells were active. The plate count on the tenth day bore out this evidence by giving a decided increase in numbers of yeast colonies over those of the fifth day. Evidently the cider was not permanently unfermentable nor was it totally immune to bacterial growth. It was thought that the generations of yeast had perhaps not been given sufficient time to develop the necessary amount of toxic substances and so it was decided to repeat the experiment in a somewhat modified way.

In the second experiment two 4-liter flasks were filled with 3.5 liters of freshly pressed cider and heated for one hour at 45° C. The coagulated material resulting from the heating was allowed to settle and was then separated by decantation. The supernatant liquid and sediment were analyzed separately for nitrogen and phosphorus as in the foregoing experiment. One of the flasks containing the supernatant liquid was inoculated with 2 per cent of a heavy suspension of the natural cider flora at its maximum activity in cider and the other with a heavy suspension of *Saccharomyces valesiacus* as in the first experiment. The procedure from this point on was similar to that already described except that the development of the yeast, and consequently the fermentation, was allowed to continue a longer time for each generation before being checked by heating.

TABLE 3.—*Analyses of cider and sediment in the second experiment at different stages of the treatment*

Treatment	Quantitative analysis in percentages						Quantitative analysis [a]			
	Supernatant liquid				Sediment		Supernatant liquid			
	C_2H_5OH in weight	Acidity in terms of CH_3COOH	Nitrogen	Phosphorus	Nitrogen	Phosphorus	Nitrites	Nitrates	Ammonia	Orthophosphates
Heated fresh cider	0.0	0.43	0.0014	0.0029	0.0196	-------	−	−	+	+
Cider inoculated with natural yeast flora:										
First inoculation	.0	----------	.0	-------	.093	-------	−	−	+	+
Second inoculation	----------	----------	.0	.0	.101	-------	−	−	+	+
Third inoculation	1.6	.42	.0	-------	.0	-------	−	−	+	+
Cider inoculated with S. valesiacus:										
First inoculation	.0	----------	.0	-------	.103	-------	−	−	+	+
Second inoculation	----------	----------	.0	.0	.098	0.0105	−	−	+	+
Third inoculation	1.6	.43	.0	-------	.0	-------	−	−	+	+

[a] +=positive test; −=negative test.

At the fourth reinoculation the flasks were incubated at 25° C. and plate counts for yeast were made 24 hours later. The cider was then bottled in pint bottles and sealed by means of metal caps. Two

bottles taken from the cider inoculated with the natural flora and two from that inoculated with *Saccharomyces valesiacus* were pasteurized for 30 minutes at 80° C. and after cooling were placed in storage in the 25° incubator with the rest of the bottles. Two months later and again four months later agar plates were prepared for yeast counts of both the pasteurized and unpasteurized bottles, and at the same time determinations for alcohol, nitrites, nitrates, ammonia, and orthophosphates were made. The data recorded in Tables 3 and 4 show that the results of this experiment were quite similar to those of the first experiment.

TABLE 4.—*Determination of active yeast cells, ammonia, orthophosphates, and per cent of alcohol in cider at different periods of incubation after the fourth inoculation with yeast in the second experiment*

Treatment	Living yeast cells per cubic centimeters [a]	C_2H_5OH	Nitrites	Nitrates	Ammonia	Orthophosphates
Cider inoculated with natural yeast flora:						
24 hours after fourth inoculation	300,000	1.60	−	−	+	+
After 2 months' incubation at 25° C	9,850,000	1.80	−	−	+	+
After 4 months' incubation at 25° C	1,700,000	2.80	−	−	+	+
Cider inoculated with S. valesiacus:						
24 hours after fourth inoculation	930,000	1.60	−	−	+	+
After 2 months' incubation at 25° C	240,000	1.87	−	−	+	+
After 4 months' incubation at 25° C	25,000	2.70	−	−	+	+
Cider inoculated with natural yeast flora of cider and that inoculated with S. valesiacus, but Pasteurized 24 hours after fourth inoculation:						
24 hours after fourth inoculation	0	1.60	−	−	+	+
After 2 months' incubation at 25° C	0	1.60	−	−	+	+
After 4 months' incubation at 25° C	0	1.60	−	−	+	+

[a] Each figure in this column is the average of counts taken from 4 plates, duplicate plates being prepared from 2 bottles.

There was a natural increase in alcoholic content due to the extra time each generation of yeast was allowed for development. However, this greater development of the several generations of yeast did not have the effect that was hoped for, namely, that of making the cider permanently unfermentable. Only the pasteurized bottles proved to be inactive. In all the other bottles fermentation took place slowly but continuously during the four months the cider was kept in storage. The renewed activity of the yeast probably began about the second week of incubation as in the first experiment. Although fermentation progressed to a certain extent the conditions for growth were never favorable. This was especially true for *Saccharomyces valesiacus*, as proved by the rapidly decreasing number of living cells during the four months of incubation. The yeast of the natural flora of the cider, probably composed of more than one species, behaved somewhat differently. The percentage of inoculum at the fourth inoculation being the same for the two yeasts, the concentration of the living cells might be expected to be similar also. But this was not the case, for the plate count showed that the number of living yeast cells was much smaller in the cider inoculated with the natural flora than in that inoculated with *Saccharomyces valesiacus*. However, as the time of incubation advanced the concentration of living cells became greater in the former than in the latter, indicating

that certain species of yeast are more resistant to the effect of their by-products than others. But, on the other hand, the amount of alcohol produced by the two kinds of yeast was approximately the same though the number of living cells varied widely. The reason for this may be explained by the fact that the members of the species *Saccharomyces valesiacus* which are known for their high alcohol production are probably more efficient alcohol producers than the members of mixed species generally found in cider. Furthermore, the number of the growing cells which appeared on the agar plates are not necessarily an indication that all these cells were active under the extremely unfavorable conditions of the cider. Many may have been inactive in the cider and the alcohol produced may have been the result of the activity of comparatively few cells in both cases.

It is interesting to note that the number of living cells in both kinds of yeast declined rapidly during the last two months of incubation. It may be that the increasing pressure resulting from continued CO_2 production in the sealed bottles was a contributing factor. Or possibly the mineral food shortage became more intense as the fermentation progressed. Either or both of these factors may reasonably be expected to exert some influence on the development of living cells. In order to ascertain to what extent they were responsible for the conditions in the bottled cider the following experiment was prepared:

Fourteen 250 c. c. cotton-plugged Erlenmeyer flasks were sterilized and after being cooled 12 of them received 100 c. c. each of the cider that had been pasteurized at the fourth inoculation of the previous experiment and was known to be free of living yeast cells. The two remaining flasks received 100 c. c. each of sterilized freshly pressed cider. The flasks were arranged in 2 series of 7, each series containing 1 flask of the sterilized fresh cider and 6 of pasteurized cider that had been treated by successive generations of yeast. One series was inoculated with 1 c. c. quantities of cider from the sealed bottles treated with the natural yeast flora of cider in the previous experiment and the other series was inoculated with 1 c. c. quantities of cider from the sealed bottles treated with *Saccharomyces valesiacus* in the previous experiment. All this was done aseptically to eliminate possible errors from contaminations. The cotton plug in each flask was then replaced by a sterilized rubber stopper through which one end of a glass tube bent at right angles was passed. The other end was connected to a similar tube inserted in N/1 KOH in an absorption tower for the purpose of collecting the CO_2 produced. The two series were further treated as follows:

The flask containing the sterilized fresh cider and the first flask containing the pasteurized treated cider in each series were not treated and served as controls.

The second flask of pasteurized treated cider in each series received 0.1 per cent peptone.

The third flask of pasteurized treated cider in each series received 0.05 per cent of ammonium nitrate.

The fourth flask of pasteurized treated cider in each series received 0.1 per cent secondary potassium phosphate.

The fifth flask of pasteurized treated cider in each series received 0.05 per cent ammonium nitrate and 0.1 per cent secondary potassium phosphate.

The sixth flask of pasteurized treated cider in each series received 0.1 per cent peptone in addition to 0.05 per cent ammonium nitrate and 0.1 per cent secondary potassium phosphate.

The added materials were thoroughly mixed with the cider in the flasks and the two series, properly arranged and connected, were incubated at 25° C. for two weeks. At the end of the incubation period each flask was thoroughly shaken to expel the gases absorbed in the cider and the CO_2 collected in the absorption towers was determined by double titration, thymol blue and brom phenol blue being used as indicators. The data obtained are given in Table 5.

TABLE 5.—*The effect of peptone and mineral food elements on the activity of yeast in cider rendered temporarily unfermentable by successive generations of yeast*

Treatment	Per cent of alcohol and milligram of CO_2 in 100 c. c. after 2 weeks' incubation at 25° C.			
	Inoculated with natural flora from treated cider		Inoculated with S. valesiacus from treated cider	
	CO_2	C_2H_5OH	CO_2	C_2H_5OH
Pasteurized treated cider	57		101	
Freshly pressed sterilized cider	5,394	5.61	5,614	5.94
Pasteurized treated cider+0.1 per cent peptone	2,824	5.53	2,270	5.40
Pasteurized treated cider+0.05 per cent NH_4NO_3	2,626		1,408	
Pasteurized treated cider+0.1 per cent K_2HPO_4	272		365	
Pasteurized treated cider+0.05 per cent NH_4NO_3+0.1 per cent K_2HPO_4	2,930	4.96	2,345	5.29
Pasteurized treated cider+0.05 per cent NH_4NO_3+0.1 per cent K_2HPO_4+0.1 per cent peptone	3,066	5.61	3,014	5.37

The active fermentation in the sterilized freshly pressed cider offers conclusive evidence that both kinds of yeast resumed their normal activity when exposed to the proper media. The data also indicate that the pressure existing in the sealed bottles as a result of CO_2 production had little or nothing to do with the rapidly decreasing number of living cells in the sealed bottles during the last two months of incubation. The addition of phosphates alone did not increase the activity of either kind of yeast, but the addition of ammonium nitrate caused a slight increase, showing that nitrogen was a limiting factor. When both ammonium nitrate and secondary potassium phosphate were added there was a decided increase in CO_2 production, indicating that one of the causes responsible for the limited activity of the yeast in the sealed bottles was lack of mineral food. The reason that more CO_2 was produced by the addition of peptone than by the addition of ammonium nitrate was perhaps that the organic form of nitrogen was better assimilated and more suitable for yeast growth than the inorganic form or that traces of phosphates were present in the peptone. The presence of traces of phosphates was indicated by a positive test for orthophosphates.

If the results of this experiment alone are considered it might be assumed that the extremely limited action of yeast in cider in which several generations had developed was chiefly due to lack of food. This assumption is supported by the fact that the addition of suitable forms of nitrogen and phosphorus caused a spontaneous and vigorous resumption of fermenting activity which resulted in a yield of alcohol approaching the normal amount in cider. But opposed to this assumption is the fact that the qualitative test for ammonia and

orthophosphates was, as far as could be observed, identical at the beginning and at the end of the incubation period, while the number of living cells seemed to increase at the beginning but showed a rapid decrease at the end of the incubation period. Granting that the food supply was constant throughout the incubation period, it seems that the number of living cells should also have been approximately the same during that period if the food supply was the only limiting factor. It would therefore appear that other causes were partly responsible. The accumulation of toxic products, generally assumed to be derived from the by-products of metabolism or from disease conditions, but as yet poorly understood because of their complexity and the lack of adequate methods, was perhaps a contributing cause. It is true that the effect, if there was any, was ultimately of minor consequence, as is shown by the final high yield of alcohol, but whatever effect there was may have been counteracted totally by enzymic autofermentation. These factors will be considered more fully in a succeeding experiment.

Two important observations not shown by the data presented, but valuable from the standpoint of manufacture, were made in these experiments and should not be overlooked. One is that the heatings required in the process caused complete coagulation of the material in suspension, resulting in a clear and attractive cider without the usual slow and tedious operation of filtering. The other is that as far as could be ascertained by taste the heatings at this low temperature did not affect to any perceptible degree the natural flavor of the cider. The first is very important from the standpoint of cost of manufacture and the second is valuable from the standpoint of quality of the product.

EFFECT OF THE FILTRATE OF THE TREATED CIDER ON ALCOHOL PRODUCTION AND ON THE DEVELOPMENT OF YEAST

Since yeast enzymes are able to ferment the same sugars that the living cells secreting them ferment, it is possible that some of the alcohol in the stored cider was caused by autofermentation of zymase freed by the disintegration of dead yeast cells. As the temperature used in heating the cider for the purpose of checking the growth of the several generations of yeast was too low to inactivate the enzymes, many of them might be liberated at each heating by maceration, thus giving them an unusual opportunity to exert considerable influence on the fermentation. The extent of this influence is difficult to determine correctly because these free enzymes are not easily obtained in a pure state. The filtering membranes of the porous candles generally used for this purpose may or may not permit their free passage, depending on the electrical charges of the filter. However, the pressure commonly used on these filters counteracts the effect of the opposite electrical charge of the filtering membrane to a certain extent and forces some of the enzymes through the openings irrespective of electrical charges. Thus approximate results may be obtained by the use of the porous candle. In this experiment a portion of each of the two differently treated ciders used in the second experiment was filtered through a sterilized N. Berkefeld candle. Duplicate 100 c. c. quantities of each filtrate were mixed with 100 c. c. of Buchanan solution previously sterilized in 250 c. c. Erlenmeyer flasks. The Buchanan solution was added to provide mineral food substances which might be lacking in the filtrates. Special emphasis

was given to soluble phosphates, as this substance seems to be necessary and is known to greatly accelerate the autofermentation of enzymes. According to Harden [5] autofermentation in media poor in phosphates may be increased from 10 to 150 per cent by the addition of suitable forms of phosphates. After thoroughly mixing the contents in the flasks, each flask was connected to an absorption tower in the manner described in the previous experiment and was incubated at 25° C. for two weeks. At the end of the incubation period the flasks were shaken vigorously to liberate any gases that might be absorbed in the solution and force them into the absorption towers to be taken up by the KOH solution. Determinations for CO_2 were made by the method previously used.

The amount of CO_2 formed was found to be so small as to be insignificant, indicating that autofermentation by enzymes was absent in the filtrates and supporting the assumption that the alcohol present in the treated ciders was probably the result of the usual activity of living yeast. As pointed out before, even this activity was very limited, as only about 1 per cent of alcohol by weight was formed during four months of incubation at the optimum temperature for yeast growth. Why the amounts of alcohol were approximately the same in both of the treated ciders irrespective of the wide variation in numbers of living yeast cells between that inoculated with the natural yeast flora and that inoculated with *Saccharomyces valesiacus* is not clear. It has many times been observed that certain individual cells of a given species of microorganism are able to perform their activities apparently unaffected in culture media which are toxic and prove to be decidedly destructive to the large majority of the cells. Likewise, certain cells of a given species have often been known to acquire a resistance to disease conditions, such as bacteriophage, to the point that they are able to grow and reproduce normally in the infected media while the majority of the cells are dissolved by the bacteriophage principle. Perhaps this same principle of adaptation or resistance is applicable to media with limited amounts of food supply. Thus it may be supposed that the majority of the yeast cells in the treated cider were unable to absorb sufficient food for growth and reproduction and finally died, while a few of the more resistant cells were able to adjust themselves to these unfavorable conditions and performed their normal activity successfully. Consequently, many of the yeast cells which were capable of growth and reproduction when plated out on favorable media were unable to perform their normal function of converting sugars into alcohol when exposed to the existing unfavorable conditions in the treated ciders. The result naturally followed that the alcohol formed was the product of the activity of comparatively few yeast cells. The fact that by far the larger part of the alcohol was produced during the last two months of incubation when the largest reduction of living cells was in evidence, seems to lend support to this supposition. That the limited activity of the yeast during the incubation period was mainly due to the limited food supply was demonstrated by the extent of the renewed activity resulting from the addition of suitable food substances and also by the final normal yield of alcohol. But, nevertheless, there is a possibility that accumulated toxic products or bacteriophage had some effect on the yeast development.

[5] HARDEN, A. ALCOHOLIC FERMENTATION. Ed. 3, p. 57-58. London, New York, [etc.], 1923.

The effect of the suspected toxic products of the treated cider on yeast development was determined by an experiment in which 10 per cent of the filtrates of the treated ciders was added to the decanted supernatant liquid of fresh cider heated at 45° C. for one hour. The filtrates were obtained as in the previous experiment and were added at the first inoculation together with the usual 2 per cent of inoculum. The filtrate obtained from the cider treated with the natural flora of yeast was added to the fresh cider inoculated with the natural flora of yeast, while the one obtained from the cider treated with *Saccharomyces valesiacus* was added to the fresh cider inoculated with *S. valesiacus*. Following this treatment the experiment was conducted exactly like the second experiment, but while it was in progress careful observations were made of the behavior of the fermentation and of the growth of yeast. The entire behavior proved to be similar in all respects to that of the second experiment and because of this similarity the data are not given. The results showed that the addition of 10 per cent of the filtrates did not have any retarding effect on the development of yeast.

Determinations for the bacteriophage principle were made by filtering through sterilized N. Berkefeld candles several samples of cider of the first and second experiment after the third and fourth inoculations. Quantities of 1 c. c. of these filtrates were added to bouillon and plain agar tubes containing 8 c. c. of the respective media. These were seeded with 1 c. c. of a heavy suspension of *Saccharomyces valesiacus* and the agar tubes were used to pour plates. The plates and the bouillon tubes were incubated at 25° C. for a week and were examined carefully at short intervals for any dissolving action of bacteriophage. The experiment was repeated several times, but at no time could any lytic or solvent action be observed either in the plates or in the bouillon tubes, thus indicating that bacteriophage was not present in the cider and consequently had no effect on the yeast development.

DISCUSSION

The results of the preceding experiments show the possibility of rendering cider temporarily unfermentable by growing successive generations of yeast and also the probability that the alcohol content produced during the process can be kept within the limits prescribed by the law for unfermented fruit juices. It was found that the factors chiefly responsible for the unfermentable condition of the cider are not the accumulated toxic products of preceding generations or the effects of disease conditions of the yeast itself, but the lack of nitrogen and phosphorus. The reason that the fermentation in the treated cider resumed a limited activity under optimum conditions for yeast growth after a week of apparent inactivity is probably due to the fact that the small traces of mineral food present in the cider were sufficient to support the growth and reproduction of a few yeast cells. That these traces of nitrogen and phosphorus did not seem to disappear with the subsequent limited yeast activity may be explained by the supposition that through the process of hydrolysis of the dead cells new supplies of available forms of these elements sufficient to maintain the life processes of a comparatively small number of cells were constantly being provided. Complete removal of nitrogen and phosphorus or even of phosphorus alone would very

likely result in a permanently unfermentable product. It is generally agreed that the development of yeast is impossible in the total absence of phosphorus, and Harden[6] states that fermentation by zymase should not occur in the total absence of phosphates.

In this study only partial success in removing either the nitrogen or the phosphorus was obtained, but it should be remembered that the work was preliminary and that further research may accomplish much. The results obtained seem to indicate that the best hope for success in this process lies in the total removal of phosphorus, because it prevents both fermentation and autofermentation. If this could be accomplished successfully the process would offer many obvious advantages. Manufacture would be simplified by eliminating the slow and tedious operations of filtering, but would nevertheless give an attractive, clear beverage possessing the natural flavor of the unprocessed freshly pressed cider. The elimination of the filtering operation would greatly reduce the cost of the initial capital outlay for machinery, thereby making possible the building of small plants at centrally located points and thus vastly diminishing the cost of transportation of the bulky raw material. In this way both manufacturer and producer would realize better returns, and a large per centage of the cull fruit now going to waste could be utilized in the form of a healthful refreshing beverage.

SUMMARY

A preliminary study was made of the effect of successive generations of yeast on the alcoholic fermentation and the beverage quality of cider.

The results showed that the nitrogen and phosphorus content of cider was low and that all but traces of each were readily removed by coagulation at a temperature of 45° C. and the growth of two or three generations of yeast.

Qualitative analyses indicated that nitrogen was at no time present in the nitrate or nitrite form, but was always present in small amounts in the form of ammonia. A positive qualitative test was at all times obtained for orthophosphates.

Clear cider, containing 0.53 per cent of alcohol and possessing the natural flavor of unprocessed, freshly pressed cider, was obtained in a temporarily unfermentable form by the growth of three generations of yeast.

The chief factors responsible for the temporary unfermentable condition of the cider were found to be the lack of nitrogen and phosphorus and not the effect of accumulated toxic products or of disease conditions of the yeast.

It was observed that the temporarily unfermentable cider was subject to a limited amount of fermentation after seven days of incubation at the optimum temperature for yeast and that this was probably due to the presence of traces of available nitrogen and phosphorus which were sufficient to maintain the life processes of a small number of yeast cells.

[6] HARDEN, A. Op. cit.