pyrethrins per ml. Exactly 1 ml. of this solution is then taken for colorimetric determination.

The colorimetric method can also be applied directly to solvent-free extracts, by dissolving them in 3 ml. of the sulfur–carbon tetrachloride reagent, without the presence of kerosine. Exactly 3 ml. of the sulfur–potassium hydroxide reagent are then added, the time of addition is carefully noted, and the samples are put in the constant temperature water bath, and filtered and read in the same manner as the kerosine extracts. A solvent-free extract of known composition must be used as a standard. The blank in this case consists of 3 ml. of the sulfur–carbon tetrachloride reagent plus 3 ml. of the sulfur–potassium hydroxide reagent.

Acknowledgment

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The pyrethrum flowers were obtained through the courtesy of Kaj and Poul Arends, Ecuadorean-American Pyrethrum Co.

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CITRUS WASTE UTILIZATION

Microbiological Production of Riboflavin and Citric Acid from Citrus Molasses

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Fermentation has frequently been employed as a method for utilizing agricultural wastes and by-products, which in the past have mainly been fermented to products of relatively low value, like yeast and solvent alcohols. To investigate the possibility of converting residues to more valuable materials, the fermentation of citrus molasses to citric acid and riboflavin was studied. Although unsatisfactory, even when greatly refined, for citric acid production, citrus molasses served very well as a substrate for riboflavin production by *E. ashbyii* (NRRL 1363). It was necessary to clarify the molasses and supplement with small amounts of a commercial yeast derivative to obtain maximum yields (over 0.7 gram per liter) in 7 to 9 days. Although the method is not expected to be competitive with existing primary fermentations for riboflavin production, application to the enrichment of citrus molasses for feeding seems practicable.

CITRUS FRUIT PROCESSING, for either canning or concentrate production, yields two major by-products—pulp and press juice. Waste peel, rag, and seeds, with any residual juice, are shredded, limed, and pressed to give a highly fermentable "press water" and a solid "citrus pulp." The amounts and compositions of these fractions vary greatly with the type of fruit, locality, and treatment used, but in general from half to two thirds of the fruit processed appears as primary waste products. In a series of comprehensive reviews on citrus wastes, Von Loesecke (*28, 29*) has presented a very complete summary of the origins and disposition of these wastes, including methods for their conversion to useful derivatives.

The disposition of these materials has become a real problem with the rapid expansion of citrus processing operations in Florida, Texas, and California. Early methods of disposal were often wasteful and ineffective and, with the rapid increase in waste volumes, a potential public health hazard was recognized. Present practice is to dry the solid pulp portion and evaporate the unstable press water to a "citrus molasses" sirup containing about 70% solids. Both molasses and pulp are used now mainly as ingredients of animal feed. A description, with flowsheet, of the citrus pulp and molasses process used by Sunkist Growers, Inc., was recently presented (*6*). The molasses produced was somewhat higher in protein (4 to 6%) but otherwise similar to the composition ranges for this product usually reported. Characteristic analyses for Florida citrus molasses have been presented (*7*), but the composition may vary over rather wide limits (*6, 13, 28, 29*).

Citrus molasses (or the original press water), with its high sugar content, is a potential raw material for fermentation, and a variety of microbiological processes for utilizing this material have been proposed (*28*). However, experimental

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studies have been made on only a few of the many possibilities.

Yeast production has been carried out on both press water and molasses (*11*, *13*, *27*). Varieties of *Torulopsis utilis* were used, and although good yields were obtained on a pilot plant scale, there has been no commercial application of this process so far. In growing yeast, one group (*11*) reported considerably better results with Texas molasses than with material from Florida. This result appears to be attributable to the different types of fruit originally processed, although direct evidence is lacking. Alcohol production from press water has also been studied (*13*) and a commercial application of this process in Florida has been reported (*28*). Operation of these alcohol plants depends on the alcohol price situation and is in general spasmodic.

Production of lactic acid (*28*) and vitamin B_{12} (*5*) has also been mentioned, but no details have been published.

Although the technical literature is rich in proposals for the utilization of agricultural and food processing wastes by fermentation, applications are limited. Increasing restrictions on the disposal of such matter continue to draw attention to utilization methods. Here, apparently, are ways in which the load (B.O.D.) on disposal systems can be reduced while returning usable chemical products to offset some of the treating costs. Unfortunately, the record of practice is most disheartening, for while the schemes proposed are technically feasible they are rarely economical. The main reason seems to be that they are usually based on conversion of the carbohydrates to yeast, solvent alcohols, and, less frequently, lactic acid. Although these materials are usually easily produced, they must compete with other large-volume by-products and synthetics.

On the other hand, there is the possible use of food and related wastes for the microbiological production of complex materials of greater unit value. Vitamins, antibiotics, and higher organic acids are strong possibilities, and this paper is devoted to a study of the production of two such compounds—riboflavin and citric acid—from citrus molasses. While these products avoid many of the difficulties inherent in the "yeast-alcohol" group, the prospects are not all good. Fermentation requirements are generally more exacting and recovery procedures more complex and expensive. Sterile conditions, often needed, mean higher equipment costs and closer control. These points, however, though of cardinal importance, can be considered only in the development of specific applications. Certainly the problem is worth a detailed experimental investigation.

After a rapid rise, the production of citrus molasses experienced a steady decline between 1948 and 1950, although the total fruit processed continued to increase (*29*). Since 1950 production of molasses and pulp has apparently again been rising, but the level attained in each season will always be dependent on market demands for citrus juices and on the supplies of cane and beet molasses available.

Microbiological Production of Citric Acid and Riboflavin

Citric acid, widely used in the manufacture of foods, beverages, and pharmaceuticals, has been prepared commercially by fermentation for over 25 years. Since 1927 citric acid fermentation processes have been predominantly of the "open-pan" type, using shallow layers of medium in trays. Although this technique may be considered obsolete when compared with submerged processes, it continues in use chiefly because of the large investment in existing equipment. Experience with other industrial fermentations indicates that, with continued improvement, submerged techniques will eventually replace the pan system. In fact, all new citric acid installations are believed to be tank units.

Citric acid is a product of the regulated (abnormal) metabolism of many organisms, but is most prominent in various strains of *Aspergilli*. *A. niger* is the variety most commonly used and the availability of stable strains producing high yields made it the choice for the present study.

The major industrial raw material for microbiological production of citric acid is beet molasses, but cane molasses and many other "natural" substrates have been tried with varying success. While no previous reports of the use of citrus molasses were found, it seemed logical to expect that the carbohydrates contained could be converted to citric acid, provided other nutrient conditions were suitably adjusted.

Foster (*4*) and Prescott and Dunn (*18*) have reviewed the theories of citric acid formation and general nutrient requirements and recent advances in the fermentation have been described (*8–10*, *14*, *15*). Microbiological production of citric acid is unique in its marked sensitivity to the cation content of the medium. Apparently appreciable acid formation occurs only when the normal system of oxidative metabolism is disturbed, a condition usually brought about by controlling the concentrations of specific "trace" metals known to be present in various respiratory enzyme systems. Recently Moyer (*12*) has demonstrated the effect of alcohols in the medium on citric acid biosynthesis, another factor which can apparently promote abnormal metabolism. In the laboratory, synthetic media have commonly been employed to study specific nutrient and trace element requirements, despite the difficulty in transposing such results to natural substrates.

Riboflavin is produced commercially, both in the pure form and as vitamin-rich concentrates, by synthetic and fermentation methods. Demands for these materials have increased steadily, and the extensive literature on new methods of production, and modifications of existing ones, has been thoroughly reviewed by Pridham (*19*).

Ashbya gossypii and *Eremothecium ashbyii*, two yeastlike organisms now in commercial use, were the only ones considered as potential producers of riboflavin from citrus molasses. Examination of the published nutrient requirements for other organisms revealed a considerable disparity between these and the composition of diluted citrus molasses. Adjustments in the medium to permit their employment would be so exceedingly detailed that the essential idea of waste utilization would be all but lost.

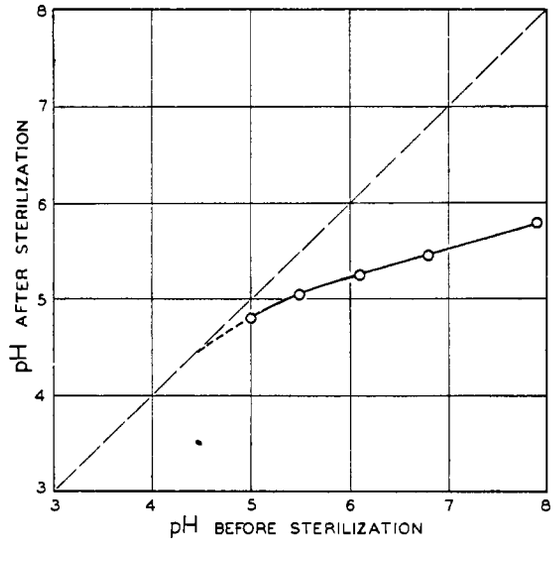


Figure 1. Relation between pH before and after sterilization in citrus molasses medium

Medium. 3.2% sugar (clarified citrus molasses)
0.3% Basamin-Busch powder
Tap water
pH adjusted with potassium hydroxide

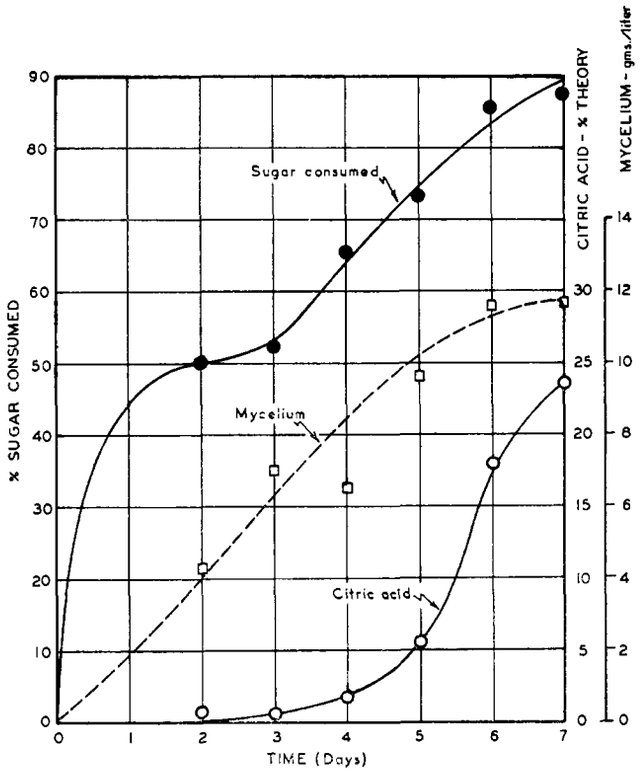


Figure 2. Citric acid fermentation of refined citrus molasses by *A. niger* (NRRL 599)—spore inoculum

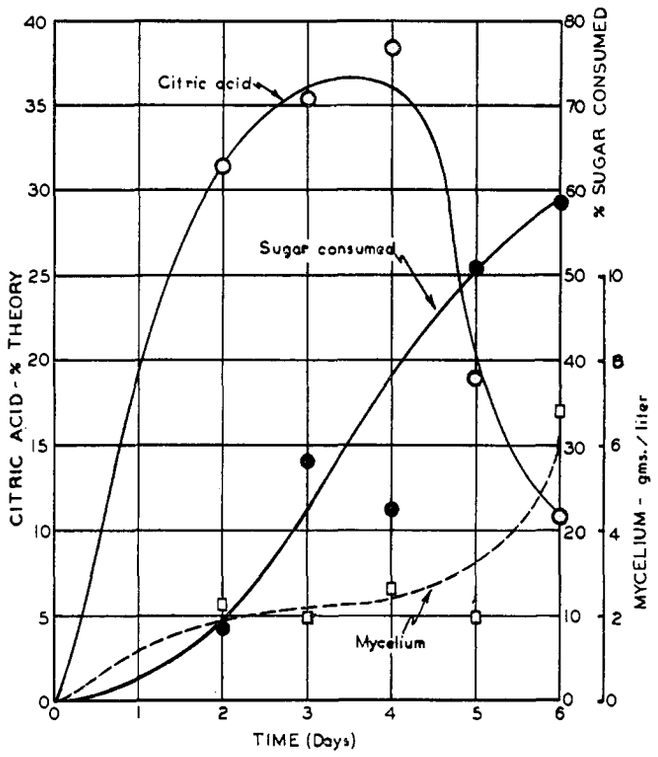


Figure 3. Citric acid fermentation of refined citrus molasses by *A. niger* (NRRL 599)—vegetative inoculum

Much recent work has been done with *A. gossypii*, the basic patents being held by the U. S. Department of Agriculture, while patents for *E. ashbyii*, combined with a variety of specific substrates in submerged aerobic fermentations, have been issued to many individuals (*8–10, 14, 19*). Many types of media have been proposed for the production of riboflavin by *E. ashbyii*, employing a wide variety of protein and carbohydrate sources (*19*). Animal proteins are included in nearly all of these formulations. Yields usually vary from 200 to 700 γ per ml., although some authors report much higher values. Tabenkin (*25*) claimed more than 700 γ per ml., Phelps (*17*) as high as 1295 γ per ml., and Moss *et al.* (*22*) as high as 2480 γ per ml. In all these studies submerged, aerobic fermentations were used, with a pH range of 4.5 to 9.0 and temperatures from 23° to 34° C. Fermentations lasted 4 to 7 days.

A. gossypii was studied by Tanner *et al.* (*26*), who employed a medium consisting of glucose, corn steep liquor, and peptone, with yields averaging 400 γ per ml. in an 8-day fermentation. Modifications of this method were described by the Northern Regional Research Laboratory group (*16, 20, 21*), with peak yields of 1760 γ per ml. reported when additional glucose was added after the fermentation had proceeded to a certain stage. The optimum pH for all fermentations reported was in the range 6.0 to 7.0 (prior to sterilization), while the temperature was 27° to 30° C.

Experimental Methods

Stock cultures of *A. niger* (NRRL 599) were maintained on Czapek-Dox agar slants, while cultures of *A. gossypii* (NRRL 1056) and *E. ashbyii* (NRRL 1363) were grown on the M-Y medium used by Pridham *et al.* (*20*). Transfers were made regularly every 7 days, the stock cultures being held continuously at 28° to 29° C.

Citric acid and riboflavin fermentations were carried out in 250- and 300-ml. Erlenmeyer flasks, respectively, with 50 ml. of medium per flask in all cases. The taller flasks were used for riboflavin experiments to lessen foaming difficulties. These flasks were shaken on a reciprocating shaker at 27° to 30° C. Unless otherwise noted, citric acid fermentations were run for 7 days.

A suspension of *A. niger* spores in sterile water, containing 0.1% detergent (Duponol), was used to inoculate citric acid shake flasks. The vegetative inoculum employed for certain experiments was prepared by inoculating a flask of regular fermentation medium with spores and allowing it to shake until mycelial growth was evident (24 to 36 hours).

Riboflavin media were generally seeded with a 24-hour vegetative inoculum grown on a medium consisting of:

Clarified citrus molasses	1.5%
Yeast extract (Difco)	0.3%
Peptone	1.0%

Adjusted to pH 6.6 to 6.8 before sterilization.

Growth and production curves were obtained by preparing a series of identical flasks and removing two each day for analysis.

Citric acid fermentations were carried out on both raw and refined citrus molasses, while riboflavin experiments employed only the raw material. The refined material is a clear, light-brown sirup obtained by ion exchange treatment of the raw stuff. No analysis of it is available. Both varieties were supplied by Juice Industries, Inc., Dunedin, Fla. Some experiments were conducted with molasses in which the cation content had been further reduced by treatment with a hydrogen-cycle, cation exchange resin (Amberlite IR 105) after dilution with distilled water to a sugar concentration about one third that of the original molasses. In addition, some molasses was treated with lime by the method of Agarwal and Peterson (*1*). Each of these special molasses fractions was subsequently used to prepare media in the same manner as the raw and refined materials.

Molasses dilutions were made with tap water for riboflavin fermentations and with distilled water for citric acid experiments and are expressed in terms of the per cent reducing sugar, determined by the Shaffer-Somogyi method (*23, 24*), in the broth as finally constituted.

In some citric acid fermentations a nitrogen-phosphorus-magnesium supplement (NPM) was employed in the con-

centrations indicated by the literature (*4*). The compounds and amounts added were:

NH_4NO_3	0.225%
KH_2PO_4	0.10%
$MgSO_4.7H_2O$	0.024%

Citric acid media, with all supplements added, were adjusted to pH 2.5 with hydrochloric acid. At this value there is essentially no pH change during sterilization. Riboflavin media, on the other hand, present a different picture. For these the desired pH was obtained by adjustment with potassium hydroxide since the diluted molasses is very acid. During sterilization, however, the pH changes appreciably, depending on the initial value. The relation between pH before and after sterilization, for a citrus molasses medium typical of the ones used in these studies, is shown in Figure 1.

Acid production was determined by titration and citric acid was not determined directly. In each fermentation run, however, qualitative checks of the acid produced were made according to the method outlined by Dunn (*3*).

Riboflavin assays were made with a photofluorometer, using the 4350 A. mercury band. This method is based on the characteristic fluorescence of riboflavin at this wave length (*2*). For this assay the samples were hydrolyzed by autoclaving for 30 minutes at 120° C., in the presence of 0.123*M* acetate buffer (pH 4.5 to 4.7). After the samples were diluted appropriately with distilled water, they were compared in the photofluorometer with a standard riboflavin solution, using precautions to protect the solutions from light during handling.

In citric acid fermentations, mycelial weight was determined by filtering the contents of a flask through tared filter paper. The tissue collected was washed and dried to constant weight at 50° C. All fermentations were checked microscopically at the first indication of contamination. Unless specifically noted, values from contaminated cultures were not taken into account in computing and correlating results.

Production of Citric Acid From Citrus Molasses

Using the approximate nutrient levels indicated in the literature (*4*) as a basis, citric acid production from citrus molasses was studied in shake flask fermentations. For each flask the sugar before and after fermentation (corrected for volume changes, etc.) was determined and the theoretical acid formation calculated. All yields are reported as the per cent of this theoretically possible acid actually found by titration. No attempt was made to determine citric acid directly; total acidity was titrated with standard sodium hydroxide and all the acid yields reported may be expected to include some oxalic, and possibly other, acid. A qualitative test was made in each run as described above and in no case was more than a trace of oxalic acid indicated. As "yields" were intended only to provide an indication of conditions under which reasonable amounts of acid could be produced, titration values were deemed adequate.

A variety of pretreatments and supplements were employed with the two initial materials, raw and refined molasses. These are summarized, with the results obtained, in Table I.

Citrus molasses is clearly not a satisfactory raw material for citrus molasses production by *A. niger*, even with refinements. The total failure of raw molasses to act as a suitable substrate may probably be attributed to its high cation content and the large quantity of suspended pectins and related matter present. Lime treatment removes most of the suspended material and some of the metals, but apparently not enough.

Although the composition of the "refined" molasses sample was unknown, tests indicated appreciable amounts of copper and iron. Elimination of at least part of these metals, as well as others not known, by further ion exchange permitted some acid synthesis. Still the results were discouraging, particularly in view of the extensive treatment required to achieve this little improvement.

To examine the course of the citric acid fermentation of citrus molasses, both spore and vegetative inoculums of *A. niger* were used on the best medium found—ion-exchanged, refined molasses at 92 grams per liter, plus the nitrogen-phosphorus-magnesium supplement. The results of these fermentations are shown in Figures 2 and 3. With a spore inoculum (Figure 2), acid production is negligible for the first 4 days. After this initial period of rapid tissue formation, citric acid production begins and the rate of tissue synthesis decreases. Sugar consumption then enters a second phase, conversion to citric acid. The run was deliberately terminated before all the sugar present was utilized, in order to avoid consumption of the acid products as metabolites by the mold.

In a similar experiment (Figure 3), using a vegetative inoculum and a medium of the same composition, the initial lag in acid synthesis was largely eliminated. Citric acid yields after 2 days under these conditions were greater than those obtained after 7 days with the spore inoculum. Maximum yields were obtained at 4 days, after which the acid yield dropped off. As expected, the use of vegetative inoculum makes possible the shortening of fermentation time with increased acid yields.

Production of Riboflavin From Citrus Molasses

After preliminary experiments had shown that *A. gossypii* could be grown satisfactorily on agar slants containing only citrus molasses (2.0% sugar) with the pH adjusted to 6.6 to 6.8, and abundantly on similar slants to which 0.3% yeast extract had been added, shake flask fermentations were carried out using this organism. Practically no

Table I. Effect of Pretreatment and Supplements on Citric Acid Formation in Citrus Molasses Media

Molasses	Treatment	NPM Supplement	Concentration, G./Liter[a]	Mycelium, G./50 Ml.[b]	Citric Acid, % Theoretical[c]
Raw citrus[d]	...	No	46–184	None	None
Refined	...	No	46	None	None
			92	0.168	None
			138	0.346	None
			184	0.451	None
			197–394	None	None
Raw	...	Yes[d]	46–184	None	None
Refined	...	Yes	46	0.077	13
			92	0.175	16
			138	0.274	Over 10[e]
			184	None	None
Raw	Lime[d]	No	92	None	None
Raw	Lime	Yes	92	None	None
Refined	Lime	No	92	None	None
Refined	Lime	Yes	92	None	None
Refined	Ion exchange[d] (IR-105)	No	46	None	None
			92	None	None
Refined	(IR-105)	Yes	46	0.267	35
			92	0.149	Approx. 35[e]

[a] G./liter of reducing sugar in fully constituted medium (by analysis).
[b] Dry weight; entire flask volume used.
[c] On basis of sugar actually consumed (by analysis).
[d] See experimental methods for details.
[e] Approximate values; wide discrepancies between duplicate flask values.

riboflavin was synthesized at sugar concentrations from 1.5 to 4.25%, despite the fact that abundant growth was observed, especially in the media fortified with 0.3% yeast extract.

This abundant growth of *A. gossypii* without riboflavin synthesis is typical of the reported unsuccessful attempts to use this organism for riboflavin production in various media. For example, it was reported by Tanner *et al.* (*26*) that efforts to utilize cane, beet, or corn molasses, in conjunction with peptone and corn steep liquor, were unsuccessful.

Table II. Effect of Supplements and Inoculum History on Riboflavin Synthesis by *E. ashbyii* in Citrus Molasses Media

Molasses concentration.	3.3% sugar
pH before sterilization.	6.6 to 6.8
Fermentation time.	6 days

Supplement	Riboflavin Yield, γ/Ml.[a] for Inoculum Grown on: MY agar[b]	Citrus molasses agar[b]
0.1% yeast extract[c]	223	301
0.3% yeast extract[c]	344	342
0.5% yeast extract[c]	392	214
0.3% Basamin-Busch[d]	485	359
0.3% Basaminbact[d]	362	347

[a] Average values.
[b] See experimental methods for details.
[c] Difco Laboratories.
[d] Anheuser-Busch, Inc.

It was also found that sucrose and maltose could be employed in place of glucose only if present in relatively pure form. This is hardly the case with citrus molasses. Although it has been found (*20*) that *A. gossypii* fermentations are not adversely affected by the presence of certain inorganic ions, the tolerable limits of the trace elements are not known. It is possible that they are exceeded by the amounts present in citrus molasses and this, together with the rather strict carbon source requirements of *A. gossypii*, may account for the failure of this organism to produce riboflavin under the conditions described.

Preliminary experiments, again using citrus molasses agar slants, indicated that yeast extract is essential for growth of *E. ashbyii* on this medium. This is evidently due to the requirements of this organism for thiamine, biotin, and inositol for growth. It was found that riboflavin was synthesized in substantial amounts at sugar concentrations from 2.1 to 4.25% with 0.3% yeast extract added. The highest riboflavin yields (290 γ per ml.) were observed at the highest sugar concentration.

The next step was then to determine the most effective level of yeast extract and to examine the possibility of replacing this rather expensive material with cheaper supplements. For this purpose, Basamin-Busch and Basaminbact, two commercial enzymatic yeast hydrolyzates, supplied by Anheuser-Busch, Inc., were tested. The results are given in Table II for fermentations inoculated with *E. ashbyii* from both the standard MY slants and citrus molasses agar (containing 0.3% yeast extract) slants.

An improvement in riboflavin synthesis with increasing yeast extract concentrations was noted when the fermentations were inoculated from MY slants. When inoculations were made from citrus molasses agar slants, however, the amount of yeast extract did not appear to have a significant effect. This may be the result of adaptation of the organism to the citrus molasses environment during the several transfers prior to inoculation. In all cases the commercial yeast derivatives gave yields superior to those obtained with the laboratory material.

Although the data are too few to permit firm conclusions, the results appear to favor the MY slant inoculum, particularly at supplement levels of 0.3% and higher. Similarly, the Basamin-Busch supplement gave consistently better yields than Basaminbact. Consequently, only MY inoculum slants and Basamin-Busch supplements were used in the remaining experiments.

Citrus molasses contains a considerable amount of suspended solids, including pectins, and denatured proteins. Although the protein portion may be usable, the net effect of such materials is often inhibitory. To check this point, a solution of molasses was filtered, prior to fortification with the yeast derivative, and compared with the regular medium. It is evident from the results (Table III) that removal of the suspended matter is essential if maximum riboflavin levels are to be realized.

As filtration proved to be slow and tedious, subsequent experiments were carried out with solution prepared by diluting the raw molasses and allowing the solids to settle out. The time required for settling and decantation was less than 1 hour and this clarified molasses gave yields entirely comparable to the filtered.

A marked dependence of riboflavin synthesis on pH and molasses concentration was noted in preliminary experiments made with both agar slants and shake flasks. These variables were then studied in shake flask experiments using clarified citrus molasses fortified with 0.3% Basamin-Busch. Vegetative inoculum, grown in the manner previously described, was used in these experiments.

As expected, the pH proved to be a critical factor, with riboflavin yields reaching a maximum at about pH 7 for these conditions (Figure 4). Below this optimum value the yield dropped rapidly, but above it, in the pH range 7 to 8, the decrease was less marked. The 4.0% sugar molasses concentration was chosen for this experiment because it was typical of the levels used in this study. Data for other molasses dilutions (2.6 and 3.4% sugar), though less complete, showed the same pH effect with the optimum value always in the range 6.8 to 7.0. The pH values indicated are all prior to sterilization and their relation to the final pH of the medium may be closely approximated from Figure 1.

In a similar manner variations in the initial molasses concentration were examined and the results are given in Figure 5. The highest yields were obtained when the initial concentration was in the range 5.5 to 6.5% sugar, with 6.0% apparently the optimum. Furthermore, 9-day fermentations gave con-

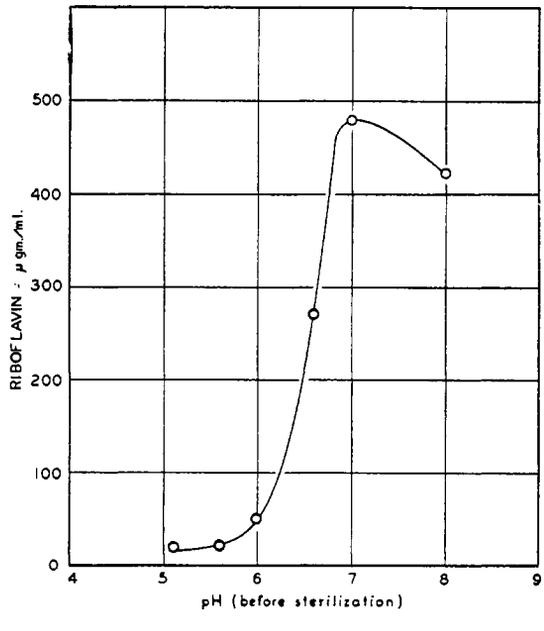


Figure 4. Riboflavin synthesis by *E. ashbyii* in relation to pH before sterilization

Medium. 4.0% sugar (clarified citrus molasses)
0.3% Basamin-Busch powder
Tap water
Inoculation. 4.0% (by volume) 24-hour vegetative inoculum
Fermentation time. 6 days

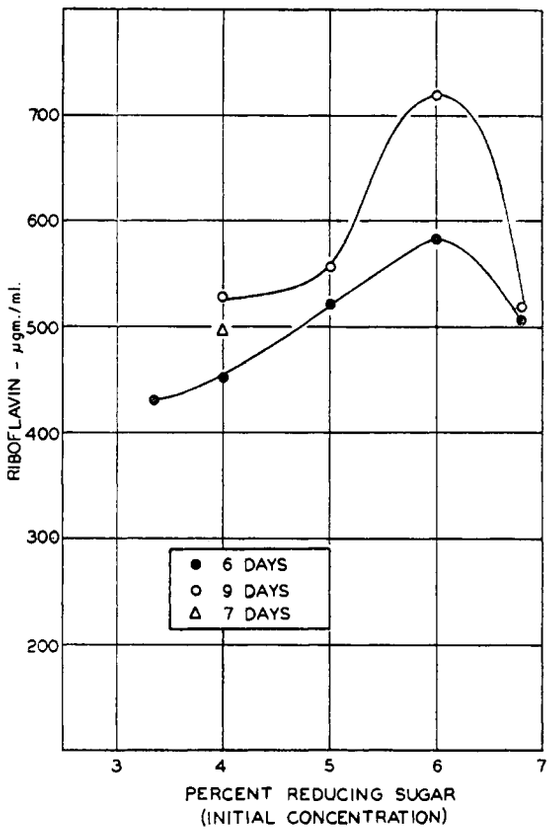


Figure 5. Riboflavin synthesis by *E. ashbyii* in relation to initial citrus molasses concentration

Medium. Clarified citrus molasses
0.3% Basamin-Busch powder
Tap water
pH adjusted to 7.8 with potassium hydroxide prior to sterilization
Inoculation. 4.0% (by volume) 24-hour vegetative inoculum

siderably higher yields than the corresponding 6-day runs. The yield of 720 γ per ml. of riboflavin obtained in this series of experiments was the highest observed in the whole study.

The effect of further supplementing the citrus molasses medium with additional protein was investigated next. Again a clarified molasses medium (4.0% sugar) with 0.3% Basamin-Busch, adjusted to pH 7 and seeded with a 24-hour vegetative inoculum, was used. The addition of 1% peptone to this basic formulation had practically no effect, giving an average yield of 515 γ per ml. compared to the control range of 480 to 520 γ per ml. Addition of 1% bone glue ("animal protein colloid") was definitely detrimental, with yields averaging only 195 γ per ml. These results are somewhat surprising, since several investigators have reported that peptone acts as a stimulant to riboflavin synthesis by *E. ashbyii*. Nevertheless, for the present case this outcome is satisfactory, for it is indicated that nitrogen sources of citrus molasses are reasonably adequate.

The course of a typical shake flask fermentation, determined by inoculating a group of flasks in common and removing two each day for analysis, is shown in Figure 6. The medium employed was the usual clarified citrus molasses (3.36% sugar) plus 0.3% Basamin-Busch with the pH adjusted to 8.0 before sterilization. This is equivalent to 5.05 after sterilization (Figure 1). A vegetative inoculum was used.

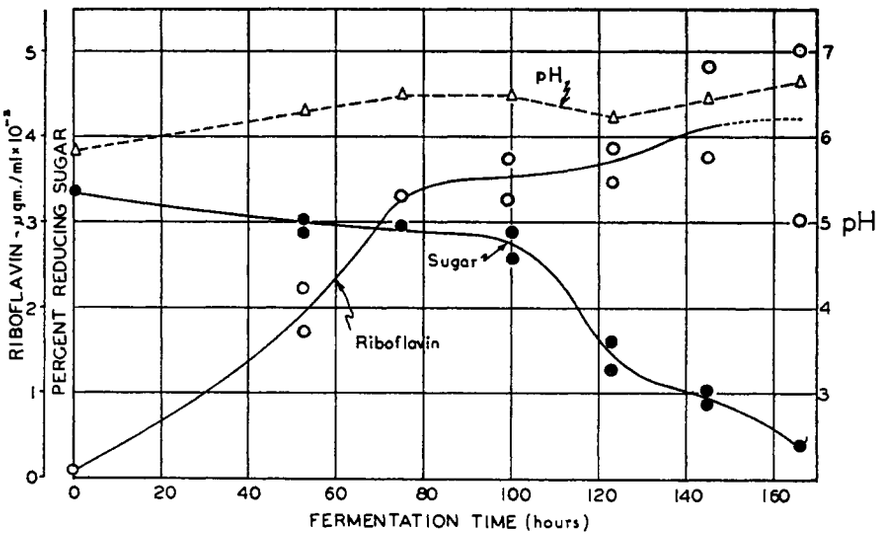
Apparently the changes in pH are related to carbohydrate utilization. Considering each period separately, it is seen that during the first 75 hours riboflavin was being produced while small amounts of sugar were consumed. As a result the pH rose from about 5.8 to 6.5. In the next period (80 to 100 hours) the two rates seem to balance each other and the pH remains nearly constant. Between 100 and 125 hours the sugar consumption is much more rapid than the rate of riboflavin synthesis and the pH drops to about 6.2. For the remaining time riboflavin synthesis appears to overcome the effect of sugar consumption and the pH rises slowly to the final value of 6.7. If the fermentation were continued beyond 6 days, higher yields could be expected.

A pH drop during the periods of rapid carbohydrate utilization is expected, but the reasons for the rising pH apparently associated with the most active periods of riboflavin synthesis are not clear. This pH rise may result from the increased utilization of proteinaceous carbon sources by the organism after carbohydrate supplies are exhausted, a commonly encountered phenomenon.

As a final step in the riboflavin study, fermentations were carried out in a 2-liter aerated flask containing 1 liter of the medium just described. About 1 volume of sterile air per volume of medium per minute was introduced through a sintered-glass disk and evaporation losses were made up by the gradual addition of sterile water. The results were similar to those shown in Figure 6; the pH held fairly constant until rapid riboflavin synthesis began, after which it rose continuously. A riboflavin titer of slightly over 200 γ per ml. was achieved in 6 days and this low value may be ascribed to bacterial contamination which occurred at about 100 hours.

Finally, a semicontinuous fermentation was carried out in the 2-liter flask by intermittent removal and addition of medium. The starting medium was again that described above with identical inoculation and aeration conditions. During the first 9 days 500 ml. of clarified citrus molasses (1.5% sugar) with 0.3% Basamin-Busch were added intermittently, and another 500 ml. of the same material, but with a sugar concentration of 3.2%, during the final 7 days. The results of this "recharge" type of opera-

Figure 6. Chemical changes in riboflavin fermentation of citrus molasses by *E. ashbyii* (NRRL 1363)



tion are given in Table IV. It was necessary to add recharge in amounts greater than the samples withdrawn in order to make up for evaporation losses.

Table III. Effect of Citrus Molasses Clarification

Supplement. 0.3% Basamin-Busch
Fermentation time. 6 days
Inoculum. MY agar slants

Molasses Concentration, % Sugar				
Unfiltered	Filtered	pH[a]	Samples	Riboflavin,[b] γ/Ml.
3.36	..	6.6–6.8	5	282
3.36	..	6.0	4	165
..	3.36	6.6–6.8	3	516
2.24[c]	1.12[c]	6.6–6.8	3	338

[a] Before sterilization.
[b] Average values.
[c] Total molasses (% sugar) same as in other fermentations, but made up of 2/3 unfiltered and 1/3 filtered material.

A peak riboflavin level of about 430 γ per ml. was attained in the 11th day and riboflavin was still being synthesized at a considerable rate after 16 days of operation. While these results are not conclusive, they are certainly encouraging and indicate that a simple recharge technique of this type offers a reasonable approach to continuous operation in this system.

Summary and Conclusions

Riboflavin and citric acid were produced on a variety of citrus molasses substrates by *E. ashbyii* (NRRL 1363) and *A. niger* (NRRL 599), respectively.

Raw molasses, under the conditions studied, is unsuitable for citric acid production. The sensitivity of *A. niger* to metal ions is so great that only a highly refined molasses can be used, and this must be treated by ion exchange and supplemented with additional nitrogen, phosphorus, and magnesium for maximum acid production. When this material, diluted to a sugar concentration of 92 grams per liter, was used with a vegetative inoculum, yields of approximately 35% citric acid on the sugar used were obtained in 4 days. In view of the high degree of purification required and the low acid yields, this method for utilizing citric molasses cannot be considered acceptable for commercial application.

Ashbya gossypii was found incapable of synthesizing riboflavin when propagated in citrus molasses media, but exactly the opposite holds true for *Eremothecium ashbyii*. Using mediums composed only of citrus molasses, clarified by decantation and supplemented with small amounts of a commercial yeast derivative, riboflavin yields as high as 720 γ per ml. of fermented liquor were obtained. Addition of protein was apparently unnecessary and in some cases detrimental.

Training the stock riboflavin cultures on citrus molasses agar slants did not give favorable results. Higher yields of riboflavin were obtained in all cases by clarifying the molasses by settling. The pH should be adjusted to 6.5 to 8.0 prior to sterilization, and the initial citrus molasses concentration should be approximately 6.0% reducing sugar. The fermentation lasts 7 to 9 days and its course is apparently indicated well by the pH changes of the fermenting liquor.

These experiments were not conducted to develop a method for riboflavin production competitive with existing processes. Rather, it was hoped that they would offer a new approach to the utilization of agricultural wastes and by-products by fermentation. While the results, indicating long fermentation times and relatively low yields, do not offer much promise for the fermentation as a primary riboflavin source, the enrichment of citrus molasses in this manner seems much more reasonable.

Table IV. Semicontinuous Fermentation in 2-Liter Aerated Flask

Fermentation Time, Days	Sample Removed, Ml.	Riboflavin Concn., γ/Ml.
6	85	284
7	65	294
9	50	359
10	50	383
11	50	432
13	50	397
16	65	366

It is also logical to expect that the results could be much improved with the use of proper fermentation equipment. Consequently, citrus molasses should not be ruled out as a raw material for primary riboflavin production on the basis of these results.

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