The Effect of Fertilization on the Glutamic Acid Content of Sugar Beets In Relation to Sugar Production

I. General Aspects

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Final waste from beet sugar factories using the Steffens process offers under favorable conditions an excellent by-product source of the dicarboxylic amino acid, glutamic acid. Concentrates of suitable Steffens filtrates can be processed to yield pure glutamic acid or the flavor intensifier, monosodium glutamate.

Studies were initiated necessarily by the tremendous variation found in the glutamic acid content of wastes from factories in different parts of the country and in different factories in the same area. It was considered advisable to learn the cause of this variation and, if possible, to determine how the glutamic acid content of wastes could be increased without detriment to the beet grower and sugar processor. Therefore, it is clearly evident that any information regarding glutamic acid must be related to its effect upon yield, sugar and processing operations if it is to be evaluated properly. For this reason our studies have been of a cooperative nature made possible by the assistance of many sugar companies, by various university workers, and by members of the Beet Sugar Development Foundation and the United States Department of Agriculture to whom we express our appreciation.

Our early studies indicated clearly that most of the differences in glutamic acid concentration of Steffens filtrates could be accounted for by differences in the glutamic acid content of the beet itself. This led to an investigation of the factors affecting the glutamic acid (or its precursors) present in the non-colloidal fraction of the beet, since only this portion reaches thin juice and may be expected to be carried over into concentrated Steffens filtrate. Work is now under way to determine the role of glutamine, the probable precursor of glutamic acid, in the growth of the sugar beet, the effects of various fertilizer treatments upon it, and the possible value of glutamic acid measurements as an indicator of the nitrogen status of the soil.

The present paper deals with analytical methods, field design and sampling techniques developed for accurate evaluation of glutamic acid, and their application to a study of the effects of fertilization on sugar beets. The responses to nitrogen fertilizer were measured at four periods of growth in a field in Yolo county, California, and the effects of manure, nitrogen and phosphorus were determined at harvest in an experimental plot on recently leveled land at Fort Collins, Colorado. Evidence is cited for considering the glutamic acid precursor, glutamine, as one of the major components of the storage nitrogen of the beet.

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Measurement of Glutamic Acid in Sugar Beets

Glutamic acid studies are complicated by the fact that three compounds are involved: *Glutamine*, found in the beet itself; *μ-pyrrolidonecarboxylic acid* to which glutamine is readily converted in solution, and *glutamic acid* which results from suitable hydrolysis of eithr glutamine or pyrrolidonecarboxylic acid.

Since all of the glutamic acid, or its precursor in the beet of interest to us, is contained in the non-colloidal fraction of the beet, some defecation treatment of whole beet material was necessary before glutamic acid determinations were made. Rasped beet pulp treated with 70% (by volume) isopropyl alcohol was found to offer a simple and convenient means of preparing extracts of beet tissue. The fraction of the total glutamic acid separated by this procedure corresponds closely to that obtained in factory thin juice. The extract after evaporation and hydrolysis is assayed microbiologically for glutamic acid.

Extraction and Hydrolysis: 25 g. of freshly rasped pulp (frozen pulp is also satisfactory) is washed into a flask with about 50 ml. of distilled water, 175 ml. of 99% isopropanol added, and the final volume adjusted with water to 250 ml. The mixture is shaken several times and allowed to stand overnight, the volume again adjusted to 250 ml. if necessary, and filtered. A 150-ml. aliquot of the filtrate is evaporated almost to dryness in vacuo. Any material sprayed on the walls of the flask or into the distilling trap is washed down into the flask with 25 ml. of distilled water, and the solution is hydrolyzed for two hours in a boiling water bath with 5.9 g. of $Ba(OH)_2.8\ H_2O$. The mixture is seeded with a pinch of barium saccharate to facilitate the precipitation of insoluble saccharate. The deposition of sugar as barium saccharate during hydrolysis minimizes destruction of glutamic acid due to browning reactions.

After hydrolysis is complete, the barium saccharate is redissolved by acidification with concentrated hydrochloric acid, and mixture adjusted with sodium hydroxide to pH 6.3 and diluted with water to a final volume of 50 ml. Barium salts which precipitate on standing in the cold are filtered off prior to microbiological assay.

Microbiological Assay: The procedure is a modification of that of Hac, Snell and Williams (1)[2] and is dependant upon the fact that the microorganism LACTOBACILLUS ARABINOSUS 17-5 requires for growth either L-glutamic acid or glutamine together with various other amino acids and vitamins. Pyrrolidonecarboxylic acid *cannot* be utilized by this organism.

The general procedures and basal medium described by Hac, Long, and Blish (2) have been modified slightly to handle barium hydrolysates of beet pulp extracts. In order to precipitate the barium salts introduced with the sample, additional ammonium sulfate is added to the basal medium used for both the standard glutamic acid curve and the samples being analyzed. Routinely, 0.035 g. of ammonium sulfate per assay tube (5 ml. final volume) is added. This amount is sufficient to prevent any inhibition of growth which would result from the barium introduced in a sample of 0.6 ml. or less. If the glutamic acid content of the hydrolysate is so low (less

[2] Numbers in parentheses refer to literature cited.

than 0.01%) that a larger volume of sample is required, additional ammonium sulfate must be added to precipitate the barium introduced: 0.1 g. for a maximum sample of 2.5 ml.

The standard glutamic acid curve is prepared in triplicate using 51 tubes over the assay range of 0.03 to 0.3 mg. of glutamic acid. Final values reported are the average of 6 determinations; duplicate tubes at 3 levels of concentration. Since the glutamic acid content of beet pulp may range from less than 0.01%, to more than 1.0%, we have found it advantageous to obtain

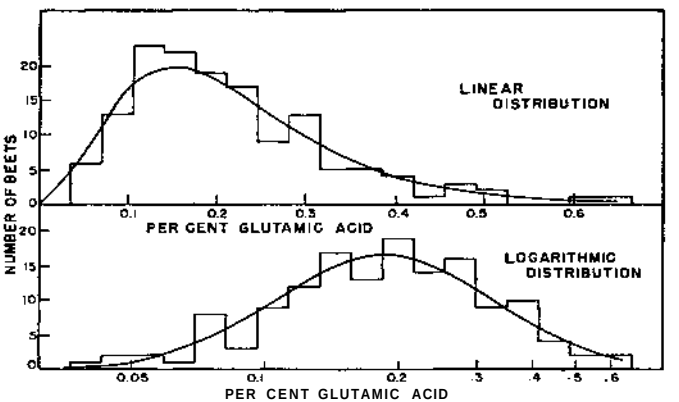

Figure 1. Distribution Curves of Glutamic Acid Concentration of Individual Beets.

an estimate of the concentration from a preliminary determination using only a single tube for assay. In this way, it is possible to select sample amounts for the final determination so that they will all fall within the most reliable portion of the standard curve, and to obtain a four fold overall spread between the 3 levels of concentration measured. That is, sample volumes would be selected to contain 0.05, 0.1 and 0.2, or 0.06, 0.12 and 0.24 mg. of glutamic acid. By this procedure, inhibitors or activators which sometimes cause difficulty in beet materials can be readily detected.

Field Techniques: Experiments designed to study responses of sugar and weight in beets are ordinarily readily adaptable to glutamic acid measurements. The chief difficulty is the wide individual variation in glutamic acid. The coefficient of variation for this substance is about twice as large as that of root weight and about five times as large as sugar content. Ideally, all beets of the plot should be included in the sample, the practical minimum

(using 5 to 8 replications) is a *forty* beet sample. Since this is about comparable to a two beet sample for sugar determinations, only very large effects can be measured. Fortunately, at least in nitrogen fertilizer experiments, the magnitude of the response is similarly inflated, so that results comparable in significance to those of sugar may be obtained.

Treatment of Data: A typical example of the distribution of glutamic acid values found in *individual* beets from the same field is shown in Fig. 1. When the glutamic acid values are grouped on a linear scale, the distribution curve is found to be considerably more pointed than a normal curve of

Table 1.—Effect of Fertilization on a Field of Sugar Beets Grown in Fort Collins, Colorado in 1947.

Treatment	Tons Beets per Acre	Tons Sugar per Acre	Percent Sucrose	Percent Glutamic Acid
Manure	16.6	2.36	14.1	0.06
P	12.1	1.72	14.2	.06
NP	18.6	2.40	12.8	.22
NPK	19.6	2.50	12.8	.25
None	8.2	1.09	13.4	.07
L.S.D. (19:1)	1.7	0.35	0.8	76%

Arrangement: Latin square
Planted: April 3, 1947
Harvested: Middle of October
Samples: 60 Beets per plot

error, and an abnormally large number of the glutamic acid values are found to be higher than the most frequent value (i.e., the curve is skewed, positively). Starting with 0% glutamic acid, the curve may be fitted accurately with a Pearson Type III curve (variance is proportional to the mean), but not with the normal frequency curve required for the analysis of variance. However, when the same data are graphed on a logarithmic scale, the distribution gives a good fit to the normal.

Transformation to logarithms is also desirable in composite samples containing up to at least 40 beets. A possible exception to its use may be in data where nearly all glutamic acid values arc over 0.3%. In such high content beets, the glutamic acid distribution curve becomes nearly normal. However, in most cases it is desirable to convert glutamic acid data to their logarithms before carrying out the analysis of variance. Consequently, results are expressed as geometric means unless otherwise noted, and differences required for significance expressed as percentage increases over the lower of the two values compared.

Fertilizer Experiments

Two field experiments, one in Colorado, the other in California, have been selected to demonstrate the effect of fertilizer upon yield, sugar and glutamic acid content of sugar beets. The *Colorado test* is taken from an experiment on restoring fertility to recently leveled land at Fort Collins conducted by Whitney, Robertson and Gardner in 1947 (3). Their results on sugar and yield were reported at the 1948 meeting of the Society. Residual samples of pulp used for their sugar analyses were frozen at the time of harvest and shipped to Woodland where they were held in frozen storage until April, 1948, when glutamic acid analyses were made.

In this experiment, fertilizer was applied at the following rates:

Treatment	Manure	P	NP	NPK	None
Lbs. N/Acre	430		430	430	
Lbs. P_2O_5/Acre	220	220	220	220	
Lbs. K_20/Acre	560			560	

The manure was applied in the spring of 1946 while the three commercial fertilizer treatments were divided equally between spring and fall applications. Very significant responses in sugar and tonnage to both nitrogen and phosphate were obtained by Whitney, et al., in the beets grown in this

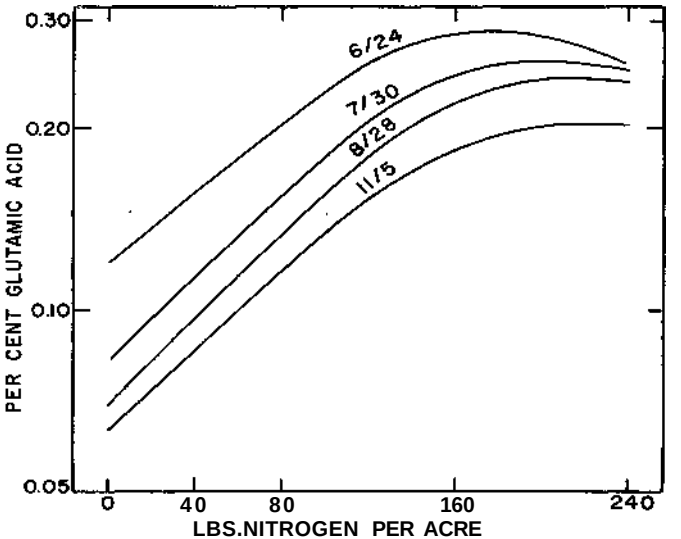

Figure 2. Glutamic Acid Response to Nitrogen Fertilization.
Glutamic acid content of sugar beets at various levels of nitrogen fertilizer at harvest (November 5) compared with three earlier sampling dates.

field in 1947. Those are compared with the glutamic acid responses in Table 1.

Beets grown with phosphate or with manure had built up no more glutamic acid than the highly nitrogen deficient untreated beets. Application of phosphate evidently increased the nitrogen foraging ability of the crop sufficiently to make possible nearly 4 tons of extra root growth, but nitrogen remained a seriously limiting factor. No response to potash was observed. The glutamic acid data indicate that the same situation holds

true for the manure treatment—the beets acquired sufficient nitrogen from the manure for 8 tons additional growth, but were still limited by nitrogen supply. This suggests that a combination of the manure treatment with additional commercial nitrogen would have given higher yields of sugar than any treatment actually used.

The treatments including commercial nitrogen fertilizer (ammonium nitrate) showed an average increase of 260% in glutamic acid content over treatments without nitrogen; a highly significant effect. Individual plots without nitrogen ranged from 0.03 to 0.19% and those with nitrogen from 0.14 to 0.35% glutamic acid. Comparison with the California data (which follows) indicates that most of the nitrogen response was attributable to the fall treatment, residual effects of the spring treatment of the previous year being only about sufficient to make up for nitrogen losses before the beets were planted.

The *California test* was a cooperative study with California Packing Corporation, the grower. Sugar determinations were made by Spreckels Sugar Company, glutamic acid by our laboratory, and petiole nitrate by Dr. Albert

Table 2.—Effects of Fertilization on a Field of Sugar Beets Grown in Yolo, California, in 1947.

rreatment bs. N/Acre	Percent Stand	Tons Beets per Acre	Percent Sucrose	Tons Sugar per Acre	Percent Glutamic Acid
0	62	13.9	16.0	2.21	0.064
40	60	15.8	16.2	2.56	.082
80	54	17.5	15.4	2.69	.114
160	54	19.2	14.4	2.76	.193
240	65	20.6	14.5	2.98	.192
240 + P	56	20.0	14.8	2.96	.215
L.S.D.(19:1)	10	1.34	0.5	0.27	24%

Ulrich of the University of California. The results of the petiole tests and a detailed description of the field have been reported elsewhere (4). The field of almost 10 acres was located a few miles north of Woodland. The design, a complete randomized block, consisted of 6 replications of the 6 treatments used; 0, 40, 80, 160, 240 pounds of N applied as ammonium nitrate, and 240 pounds of N + 200 pounds P_2O_5 (as treble superphosphate) per acre. Each plot was 12 rows wide and 600 feet long. U. S. No. 33 seed was flat planted on March 24, 1947. The field was thinned May 6, fertilizer applied as a side dressing May 7 and 8, and the beets hand harvested November 11.

Yields were taken from the entire harvest area of 12 rows per plot, but sugar and glutamic acid measurements were obtained from 40 beets sampled from the 4 center rows of each plot a few days prior to harvest. The weights of these samples were in general agreement with the harvest yields.

The results at harvest given in Table 2 showed a pronounced nitrogen deficiency in this field. Beet yield increased regularly to the highest nitrogen level tested, but the data on gross sugar production indicated that in terms of profitable return to the grower, little was gained by applications of fertilizer in excess of 80 pounds of nitrogen per acre. Phosphate treatment

was ineffective in all variables tested, and may be considered a duplicate of the 240-pound nitrogen treatment. Ulrich stated that the petiole test indicated that "the phosphate failed to get into the beets."

All analytical data on samples seem to point to a sharp cessation of response to nitrogen over 160 pounds per acre. This effect has not been duplicated in any of our other fields and appears to be caused largely by the chance occurrence of unusually fertile 160-pound plots and infertile 240-pound plots, at least in the 4 row sampling areas. On the basis of our other studies, nitrogen responses far beyond the 0.2% glutamic acid and 14.4% sugar obtained in this field should be possible.

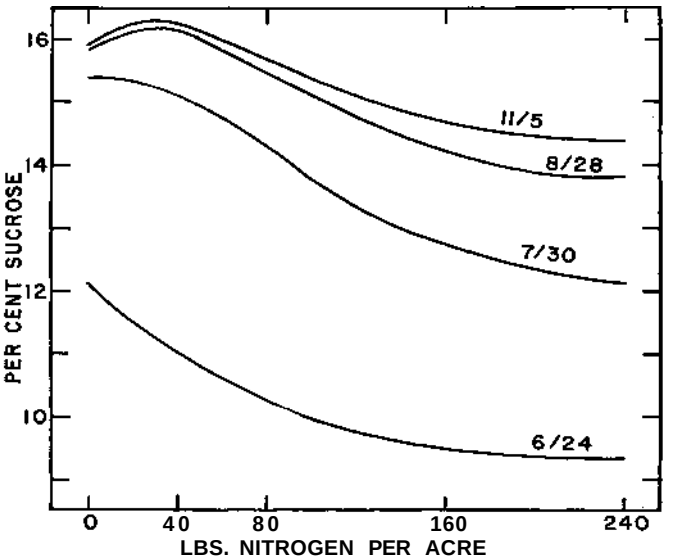

Figure 3. Effect of Nitrogen Fertilizer upon Sucrose Concentration. Effect of nitrogen fertilizer on percent sucrose at harvest (November 5) compared with three earlier sampling dates.

Nitrogen caused a rapid increase in glutamic acid content up to 160 pounds of nitrogen per acre. The average slope of the best straight line regression on nitrogen level using a logarithmic scale for glutamic acid indicated an increase of 115% for any 160-pound increment of nitrogen. The advantage of using the logarithmic scale is indicated by the range of values

of individual plots around the mean: the control treatments ranging 0.03% around a mean glutamic acid of 0.064% while the 240 plots ranged 0.200% around a mean of 0.192%. Use of logarithms gives a homogeneous variance for all treatments.

Function of Glutamic Acid

In order to obtain better insight into the function of glutamic acid (or its precursor) in the growth of the beet, three additional samplings were made in this field prior to harvest, the first on June 24, three months after

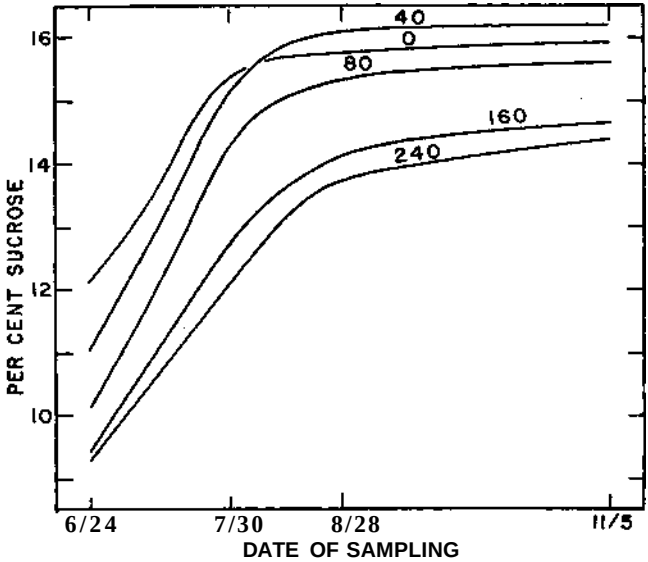

Figure 4. Effect of Age of Beet upon Sucrose Concentration at **Different Fertilizer Levels.**

Increase in percent sucrose with increasing age of the beet at different levels of nitrogen fertilization in a field in Yolo County, California in 1947.

planting; the second on July 30; and the third on August 28, roughly 2 months before harvest. The results from the 6 replications of each treatment were averaged and the best curves depicting the effects of nitrogen fertilizer on sugar and glutamic acid are shown in Figs. 2-5.

The response of glutamic acid to fertilizer nitrogen observed at harvest

was corroborated by the three earlier samplings *(Fig.* 2), but the flatter curve of the first sampling date, June 24, indicates a less pronounced effect of nitrogen level in young beets (average weight 12 ounces). The average response at 160 pounds of nitrogen was 56% as compared with 115% at harvest. This increase of slope with age is quite significant. The abnormal lack of response between 160 and 240 pounds of nitrogen in this field makes it difficult to be certain of the effect of age upon response at higher nitrogen

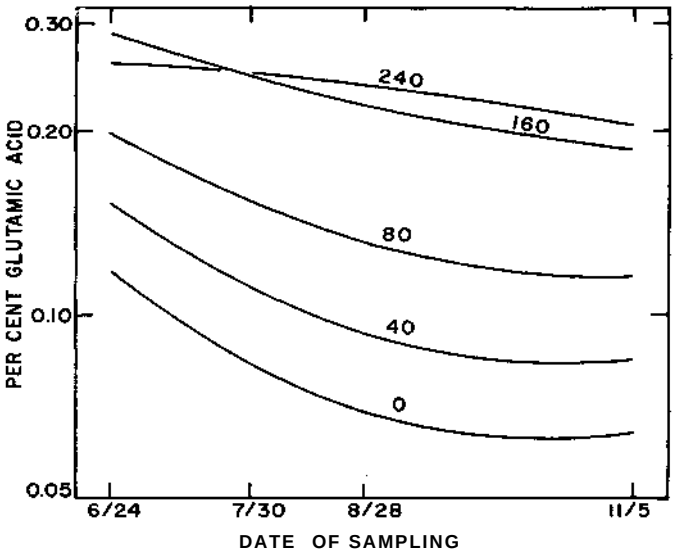

Figure 5. Effect of Age of Beet upon Glutamic Acid Concentration at Different Levels of Nitrogen Fertilizer.

levels even though a greater tendency toward straight line response (less curvature) with increasing age is evident in Fig. 2.

Comparable curves indicating the effect of fertilizer nitrogen in decreasing the sucrose percentage at the various sampling dates are described *in* Fig. 3. Here, the maximum effect was obtained at the July 30 sampling, but the relative change is much less pronounced than those at any date for glutamic acid.

Beet yields on **the** first sampling date were not affected greatly by nitrogen

fertilization, but during July and August marked differences in treatments became evident. Apparently, very little growth occurred during September and October, because the November yields were not appreciably different from those at the end of August. Actually, in the untreated plot there appeared to be a loss. These beets had practically no tops left at harvest and contained less sugar than the beets of the 40-pound nitrogen treatment, which may confirm the tendency to maximum sugar at the 40-pound treatment even though it is not statistically significant.

The expected rapid rise in sugar with increasing age of the beet, depicted more clearly in Fig. 4, shows a sharp levelling off during September when growth had practically ceased. Sugar increases with age much more rapidly than glutamic acid decreases. The cessation of growth during September was evidently not caused by lack of nitrogen except possibly in the unfertilized plots.

The regular decrease in glutamic acid with age is shown in Fig. 5. The change is small (averaging about 10% per month) and nearly disappears at the lower N levels during the two months prior to harvest. Thus, harvest date has little effect on experiments on glutamic acid. The average decrease with age is greater the lower the nitrogen fertilizer usage, but the percentage decrease with age, measured by the curvature of the line, is also greater. In fact, at the 240-pound level, the curve bends in the opposite direction.

It seems possible that the nitrogen required in formation of beet protein could be stored conveniently as the glutamic acid precursor, glutamine. Thus, under limited supplies of nitrogen, the glutamine content of young, rapidly growing beets would be expected to decrease more rapidly than would be the case with more mature beets. Lack of photosynthetic power to reduce nitrates as well as rapid growth rate probably limits the glutamine content of young beets at a relatively low value.

The view of glutamine as a storage material is substantiated by the results obtained in an experiment in which the glutamic acid content of beets which had gone to seed was compared with that of non-bolters in the same field. Individual analyses were made of 48 bolters found in a quarter-acre area in a field of U. S. No. 33 beets. A control beet with roughly the same competition was selected near each bolter and analyses made on composites of 8 beets each. Results were as follows:

	Non-Bolters	*Bolters*
Mean Root Wt. in Lbs.	2.61	1.42
Weighted Mean Sugar %	16.9	13.7
Median Glutamic Acid %	0.17	0.007

Seven of the bolters, including all those over three pounds in weight, were in the usual range of glutamic acid; that is, over 0.04%. Seven others were at 0.003% glutamic acid, the lowest limit at which the analytical method can be used. Apparently in most cases the demands of the beet for nitrogen used in protein formation for growth of the seed stalk had exhausted completely the supply of glutamic acid in the root. Further studies on the function of glutamic acid will be reported later.

Summary

Methods for measurement of potential glutamic acid in sugar beets have been described and applied to a study of the effects of fertilization. In a California experiment, fertilizer nitrogen caused a logarithmic increase in the glutamic acid of beets, more than doubling it by an increment of 160 pounds of nitrogen between 0 and 240 pounds of nitrogen per acre without impairment of sugar tonnage. A similar response was obtained *in* Colorado from commercial fertilizer, but not from manure. The addition of phosphate had no effect on glutamic acid.

Glutamic acid decreased with increasing age of the beet, but the decrease during the harvestable period was only 12%. Beets which had gone to seed contained much less glutamic acid than non-bolters from the same field. Glutamine, the probable precursor of glutamic acid, appears to be one of the major components of the storage nitrogen of the beet, and a study of this material offers information regarding the nitrogen status of the crop as well as its potential value as a by-product material.

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