

ENZYMATIC HYDROLYSIS OF STARCH IN CANE JUICE

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Abstract

The use of enzymes for hydrolysing starch in cane juices has been applied in South Africa for some years. Bacterial amylases, notably those derived from *Bacillus subtilis*, have the advantage of stability at higher temperatures. Factory trials have shown that application of less than 10 ppm of bacterial amylase can destroy over 85% of the starch in mixed juice in fifteen minutes. Applications of enzymes to syrup and clarified juice have also yielded promising results.

Historical

The process of decomposition of starch in cane juice has been applied many times during the history of sugar production.

Haddon and de Sornay reported on the high starch content of Uba cane in 1927.^{9,10} A year later Haddon reported the removal of starch from sorghum and Uba juice by the use of a malt amylase.^{11,12}

Feuilherade reported on the experimental use of an enzyme "Ubase".⁷ Though the source of this enzyme was not disclosed, it appears that the price was in the region of four rand per pound. The enzyme was apparently of low activity as it had to be used at a rate of 100 ppm on juice. Feuilherade assumed that the starch was completely transformed into glucose, and based a quantitative determination on this assumption.

Boyes² used malt enzyme for the decomposition of cane starch in juices and masseccutes, but found that the price remained prohibitive for general application.

In 1958 Nicholson and Horsley published the presence of an amylase in cane juice, and described a method for the elimination of cane starch in juice.¹⁴ These authors reported a degree of starch decomposition of 80–90%, and occasionally even higher, at a temperature of 70°C and a pH 5.7 to 5.9.

The "natural enzyme" method was used experimentally at various sugar factories in Natal and adopted as a standard practice by The Tongaat Sugar Company.³ Carter⁶ published results with this process in which 50 to 60% of the starch originally present was decomposed at a pH of 6.5 in eight minutes. In the 1967/68 season the average amount of starch decomposition was 70%.⁴ During the 1967/68 season several other South African sugar factories started to apply the natural enzyme process.

Action of Enzymes on Starch

Starch, which occurs in plant cells as granules, is a mixture of two polysaccharides. The major component, amylopectin, which represents 75 to 85% of most starches, is a multi-branched polysaccharide of molecular weight 10^7 and consists of chains of (1→4) — linked α -D — glucose residues. The chains, which contain 20 to 25 of these residues, are interlinked to form a ramified structure, by means of α -D-(1→6)—glucosidic linkages. Amylose is a linear polymer built up from several thousand (1→4) linked α -D—glucose residues. While both polysaccharides give a colour reaction with iodine, amylose is the fraction mainly responsible for the colour in the starch-iodine addition compound which is used for analysis.

Enzymes are complex structure proteins of molecular weight between 10^4 and 10^6 . They contain a number of active groups and are highly specific catalysts in numerous synthesis and degradation reactions. They are isolated from living organisms and seldom remain stable at temperatures as high as 100°C.

The amylases, one of two distinct groups of enzymes which attack the (1→4)— α -D—glucosidic linkages, may be subdivided into three main types.

α -amylases, the most widely occurring forms, catalyse a random hydrolysis of the starch, causing a rapid decrease in turbidity, viscosity, and staining power with iodine. They form D-glucose, maltose, maltotriose, and limit dextrans of a degree of polymerisation of 4 — 8, which contain the (1→6) inter-chain linkages.

β -amylases, which occur only in higher plants, catalyse a stepwise hydrolysis of alternate linkages, in an exterior chain of (1→4) linked α -D—glucose units, with the production of maltose. β -amylolysis is arrested by inter-chain linkages and other structural irregularities. Viscosity and staining power with iodine decrease much more slowly than in the previous case. The residual β -dextrans amount to 20 to 30% of the original quantity of starch.

A third group of amylases, found in some micro-organisms, liberates D-glucose as the primary product. Some of these amyloglucosidases convert starch quantitatively into glucose, and must therefore be capable of hydrolysing both the (1→4) and (1→6)

*For α read alpha.

linkages. They might be used for starch analysis. An example is the enzyme isolated from *Rhizopus delamar*.¹⁵

Enzymes can be inhibited in their action by the presence of certain other organic or inorganic components. They can also be activated by the presence of other chemical compounds.

Bacterial Amylases

The main disadvantage of the use of malt enzymes in the sugar industry is their thermal instability. For successful application cane juice must be heated to at least 70°C to dissolve the starch granules, and re-cooled before the enzyme is added. It is obvious that an enzyme capable of hydrolysing starch at higher temperatures would offer an attractive advantage.

Thermostable bacterial amylases, mainly isolated from *Bacillus subtilis*, a highly thermophilic bacterium, are commercially available in large quantities, these enzymes have been used for some time in the food industry for starch hydrolysis and in the textile industry as desizing agents.

These commercial bacterial amylases are free from invertase activity. In the presence of sufficient starch substrate they retain a reasonable activity even at 100°C. Certain metallic ions, especially copper and iron, inactivate the enzymes, while the presence of starch substrate, calcium and sodium ions protect the enzyme against inactivation.

In 1964 the Sugar Milling Research Institute carried out laboratory experiments with a bacterial amylase in cane juice.⁵ Although starch hydrolysis was found to be almost complete under optimum conditions; cost calculations showed that the process would not be economic on a commercial scale.

In 1967, Gray and Morton⁸ rekindled interest in bacterial enzymes by showing in pilot plant tests that a reasonable degradation of starch in cane juice could be obtained with small quantities of enzyme.*

Laboratory Experiments

1. Factors influencing starch hydrolysis in sugar products.

The influence of various factors on the degradation of pure potato starch by bacterial amylase (Bactamyl D-200) was studied in the laboratory.

In figure 1 the influence of pH on the starch degradation is shown. The starch was dissolved in phosphate-citrate buffer solutions of the required pH and the residual starch content determined after fifteen minutes. From the results it is evident that the reaction, which is fast below pH 6.5, stops at pH 8.0.

Figure 2 shows the hydrolysis of a pure solution of potato starch at pH 6.5 at various temperatures

* (The trade name of the enzyme used is Bactamyl D-200. The mentioning of a trade name does not infer that either of the authors of this paper, or their principals, have preference for a certain product.)

and after various reaction times. Most of the hydrolysis is completed in the first five minutes. It can be seen that the enzyme is not stable at elevated temperatures in pure dilute starch solutions. Later experiments indicated that the thermostability of cane juices was much greater.

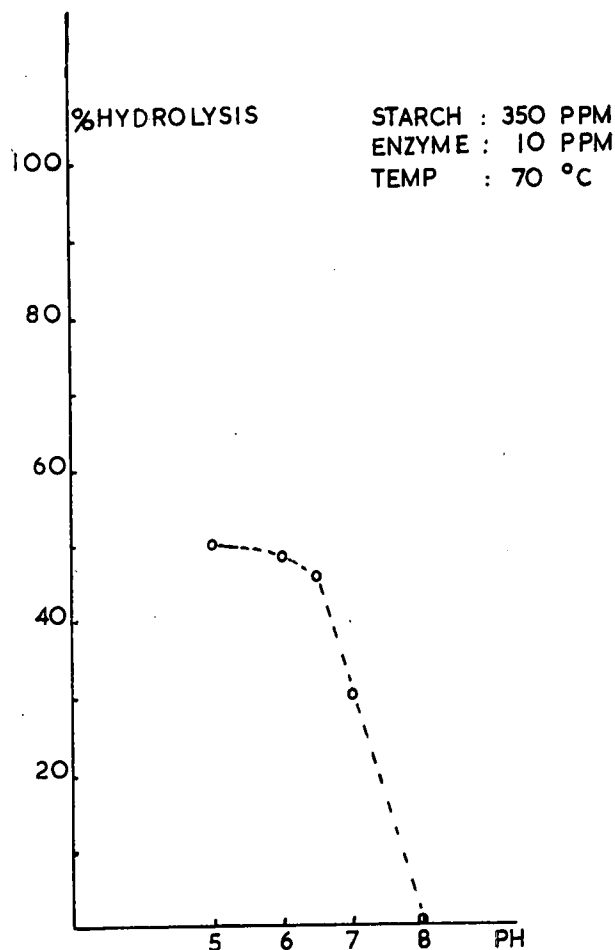


FIG. 1 STARCH DECOMPOSITION VERSUS PH

As the analysis of starch is based on the blue colour of the iodine-amylase addition compound, the rate of degradation of pure amylopectin, prepared by butanol precipitation of the amylose fraction in potato starch,¹⁴ was determined at 60°C, pH 6.5. It was found that the rate of hydrolysis of the amylopectin was approximately the same as that of the total potato starch.

It is possible that degradation of starch in mixed juice can be carried out in one step if a temperature can be found at which most of the starch in the juice is dissolved, while the enzyme retains its activity. The rate of decomposition of starch in mixed juice was determined at pH 6.5 at various temperatures. Table 1 summarises the average data from a number of experiments with juice from several cane varieties.

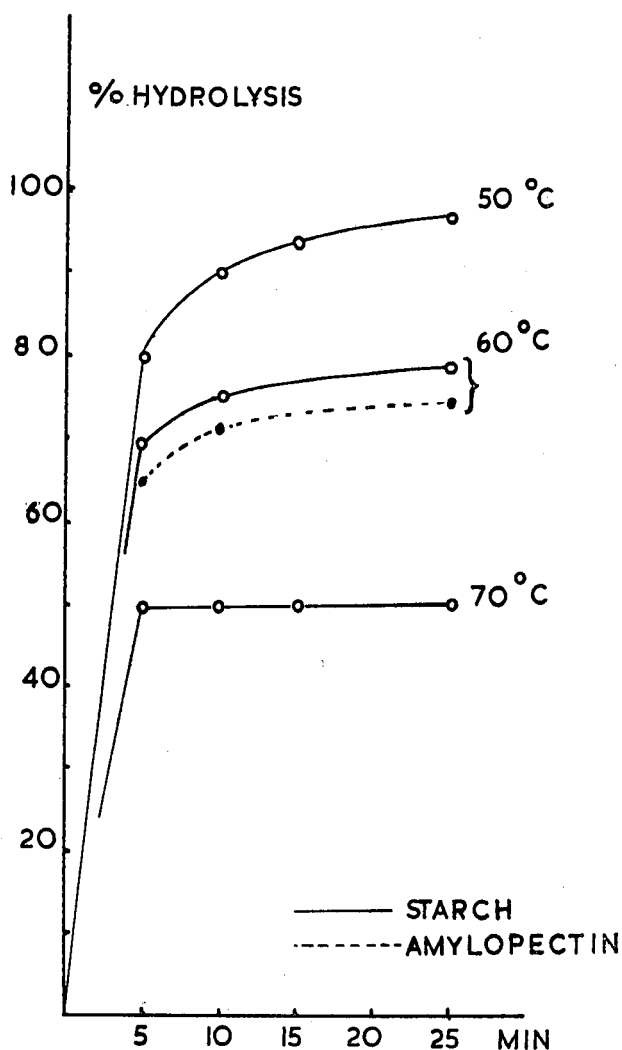


FIG.2 STARCH DECOMPOSITION AT DIFFERENT TEMP.

TABLE 1
Hydrolysis of starch in mixed juice at pH 6.5 at various temperatures with 10 ppm enzyme

Temperature °C	After 15 minutes	% Hydrolysis of starch after 30 minutes
30	80	80
60	13	36
70	46	47
77	70	80
80	80	88
83	30	30

There is an alternative, two stage method for treatment of mixed juice. The juice is first heated to a sufficiently high temperature to dissolve all the starch, and then cooled to the most favourable temperature for maximum enzymatic hydrolysis.

Mixed juice was boiled for 5 minutes at 6.5 pH, cooled, and treated with 10 ppm of enzyme. The results are shown in Table 2.

TABLE 2
Hydrolysis of starch in boiled mixed juice at pH 6.5 at various temperatures with 10 ppm enzyme

Temperature °C	% Hydrolysis after 15 minutes
60	90
70	94
80	87

It is evident that between 60 and 80°C the rate of hydrolysis of starch in unboiled juice is mainly governed by the rate of solution of the starch. The reaction rate appears to be linear with respect to temperature until 80°C. The steep drop in reaction rate above 80°C indicates that with the amount of starch present in the juice, the enzyme is inactivated at about that temperature. With greater concentrations of starch substrate the enzyme might be stable at higher temperatures.

Table 2 indicates that once the starch is in solution, the enzymatic reaction itself does not show a very pronounced temperature optimum, although 70°C appears the best under the conditions investigated. The improvement in starch decomposition by the two stage method is clear however, the percentage decomposition rising from 80 to 94% using the same quantity of enzyme. The hydrolysis of starch in cooled factory clarified juice becomes an obvious possibility.

The starch hydrolysis could also be carried out in the syrup stage, the advantages being a higher concentration of starch, most of which would be in solution. However, the higher viscosity of the syrup slows down the reaction rate, a fact mentioned by Boyes.²

In laboratory experiments with syrup at 65° brix, the maximum hydrolysis obtained was 55% in 30 minutes at 70°C using the same starch/enzyme ratio as in the experiments with cane juices. The percentage hydrolysis can be increased by adding the enzyme into a stage of the evaporators in which the temperature is not too high to cause inactivation, but the viscosity of the medium is lower than for syrup. The procedure has been applied with some success in brief factory scale tests at Amatikulu.

2. Inversion of sucrose during starch decomposition

Although the bacterial enzymes themselves do not show any invertase activity, inversion of sucrose will occur if the starch decomposition is carried out in mixed juice. Apart from chemical inversion, which depends on pH, salt concentration and temperature, cane juice contains invertase. However, the pH of 6.5 and the higher temperatures involved should reduce the inversion to a minimum.

To determine the inversion rate cane juice was adjusted to pH 6.5 and quickly heated to 80°C. When this temperature was reached the enzyme was added and the juice poured into a three-neck flask provided with a sealed stirrer and a reflux condenser. A small stream of nitrogen was bubbled through the juice to prevent any oxidation of reducing sugars in the solution. Maintaining the flask at 80°C in a thermostatically controlled water bath, samples were taken using nitrogen pressure, at the start and at half-hourly intervals. The samples were immediately cooled to room temperature and the reducing sugar contents determined by Lane & Eynon titration. From the increase in the reducing sugar content the percentage of sucrose inverted was calculated. The results are shown in figure 4.

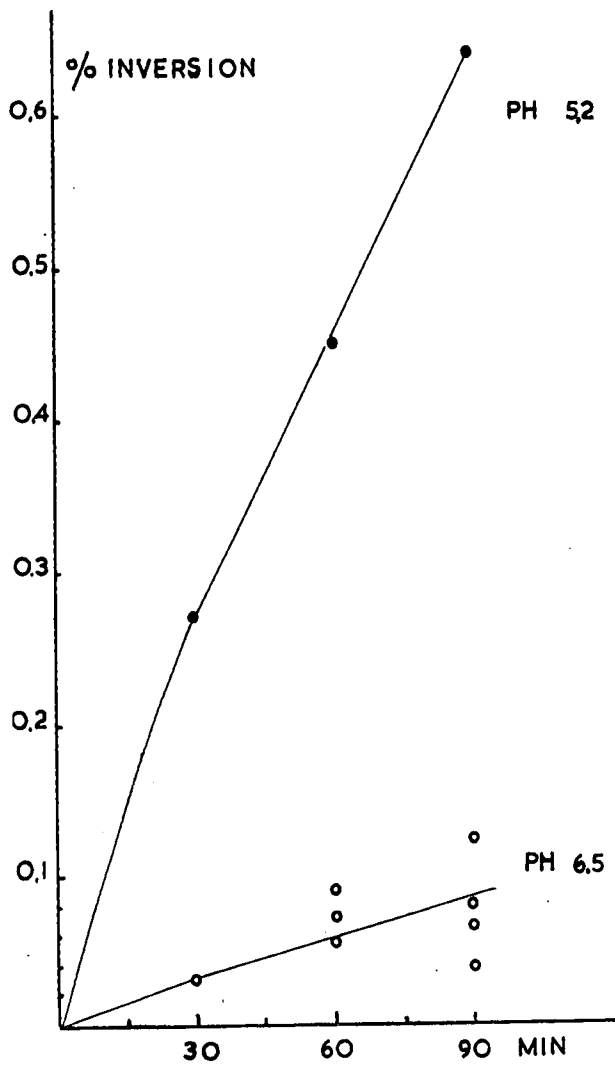


FIG 4 RATE OF SUCROSE INVERSION IN MIXED JUICE AT 80 °C

It is clear from these results that the amount of inversion in mixed juice during 15 minutes at pH 6.5 and 80°C is insignificant and may be ignored when considering practical application of the enzyme.

3. Decomposition products

As mentioned earlier, the decomposition products of α -amylolysis of starch are glucose, maltose, maltotriose and limit dextrans with a degree of polymerisation of 4 to 8. This is only true if the enzymatic hydrolysis is carried out to completion, which is not likely to be the case in an industrial application.

A good indication of the size of the eventual breakdown products can be obtained from the decrease in the colour of the iodine addition compound during hydrolysis. The eventual absence of colour with iodine indicates that the residue consists of small molecules as can be seen from Table 3.

TABLE 3

Iodine colours with glucose polymers of different degree of polymerisation¹³

Colour	Polymerisation Degree of
Red	15-21
Red-purple	30-36
Blue-green	40-45
Blue	> 50

(The degree of polymerisation of natural amylose is 10³.)

A paper chromatogram of a starch hydrolysate obtained with bacterial α -amylase, indicated the presence of a series of mono and oligosaccharides, as shown in Figure 5.

Pilot Plant Scale Investigations

The pilot plant at Mount Edgecombe consisted of five 45 gallon drums connected in series and fed from the clarifier outlets. The temperature of the juice entering the first drum could be controlled by means of a water jacket. pH was controlled by means of manual addition of hydrochloric acid combined with an in-line pH meter and recorder. Enzyme was added by means of a small peristaltic pump. The flow of juice through the system was regulated to give a retention time of twelve minutes per drum. Samples of incoming juice and juice after selected retention times were analysed for starch.

The effects of varying temperature and pH on the starch destruction are illustrated in Tables 4 and 5.

TABLE 4
Destruction of starch in clarified juice by bacterial amylase at natural pH of juice and various temperatures

ppm enzyme on Juice	Inlet pH	Retention Time in Minutes					
		12		24		36	
		Temp. °C	% Removal	Temp. °C	% Removal	Temp. °C	% Removal
10	7.7	87.5	22	86	22	84.5	22
10	7.8	78	54	77	73	76	81
10	7.2	76	67	74	87	72	95
10	7.5	74.5	70	73	89	72	100
10	7.4	67	87	66	97	—	—
5	7.7	77	36	75	54	73	64
5	7.4	73	50	71	77	69	86
5	7.4	67	58	66	80	65	89
5	7.3	71	61	69.5	79	68	90
5	7.1	74	66	72	83	71	90

TABLE 5
Destruction of starch in clarified juice by bacterial amylase at varying pH and controlled temperature

ppm enzyme on Juice	Inlet pH	Retention Time in Minutes					
		12		24		36	
		Temp. °C	% Removal	Temp. °C	% Removal	Temp. °C	% Removal
5	7.2	77	36	76	54	75	65
5	6.6	77	42	76	58	75	69
5	7.5	75	30	74	50	73	60
5	6.2	75	56	74	73	73	80
5	7.5	72.5	51	72	76	71	86
5	6.4	72.5	61	72	79	71	90

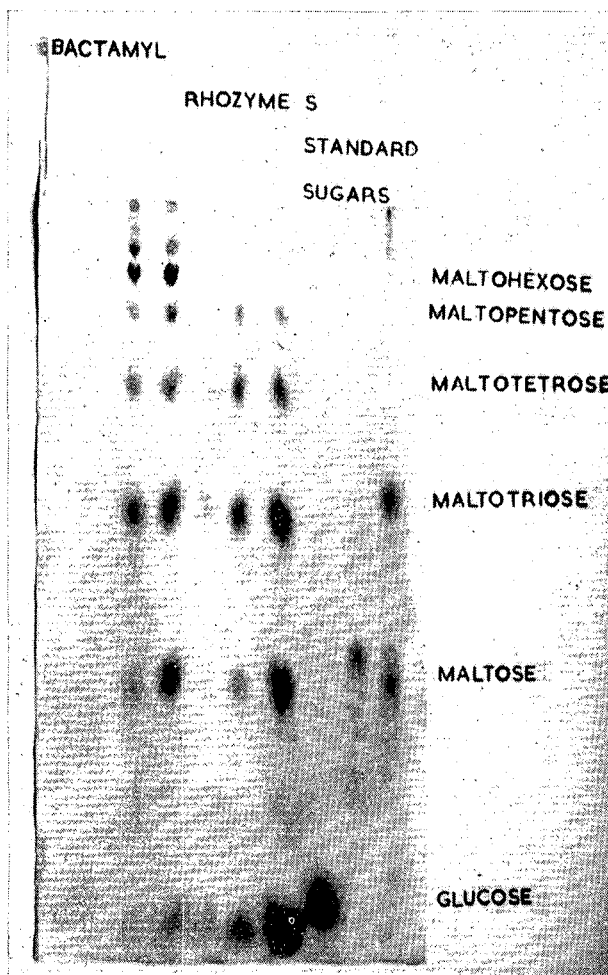


FIG. 5. PAPERCHROMATOGRAM OF A STARCH HYDROLYSTATE USING 2 DIFFERENT ENZYMES

Experiments On An Industrial Scale

1. Addition to the evaporator at Amatikulu

The twin quadruple effect evaporators at Amatikulu were an obvious choice for investigating the effect of enzyme on concentrated clarified juice.

Enzyme was dosed, at a rate equivalent to 10 ppm on clarified juice, to one of the third effects, in which the temperature ($\pm 82^\circ\text{C}$) and the brix ($\pm 30^\circ$) were closest to the optimum determined earlier in the laboratory. Samples were taken simultaneously from

the outlet of each side, and the starch destruction by the enzyme determined from the analyses of the two syrups.

The average destruction was around 70% with a maximum of 75%. Investigations were halted by the ending of the season.

2. Addition to mixed juice at Melville

Mixed juice was heated in the primary juice heaters to $78-80^\circ\text{C}$, automatically controlled by regulating the steam supply to the heaters. While this control did not cope very well with surges in juice flow, the temperature in the retention tanks remained reasonably stable.

The combination of Oliver filtrate with the main stream raised the pH to 5.6-5.7. No means were available for maintaining the pH at exactly 6:5.

A 3% aqueous solution of bacterial α -amylase (Bactamyl D-200), was added at the required flow rate by means of a peristaltic pump, and the juice passed through retention tanks with an average retention time of twelve minutes. After the retention tanks the juice was limed and clarified by conventional defecation.

Starch analyses of mixed juice and clarified juice were carried out at regular intervals, while daily composite samples of raw sugar were analysed before and after affination.

3. Addition to Mixed Juice at Darnall

Combined mixed juice and Oliver filtrate was heated to a controlled 80°C and limed to 6.5 using the pH control installed for vacuum flotation. A dilute aqueous solution of Bactamyl D-200 was added via the mono-pump normally used for dosing flocculant and the juice drawn into the vacuum flotation tank. Following an estimated fifteen minutes retention time, the juice was relimed and clarified.

One disadvantage of the procedure used was the extra load placed on the Bach clarifiers. Under normal conditions at Darnall the vacuum flotation system removes a large proportion of the mud and the demands placed on the subsidiers are small. When vacuum flotation was replaced by enzyme treatment all clarification had to be done by the undersized Bachs. The overload produced considerable carry-over which resulted in high ash and insolubles contents in the raw sugar.

TABLE 6
Industrial Experiments at Melville Sugar Estates, pH
during enzymes processes 5.6 to 5.7

Period 1968	Process Applied	Enzyme Added ppm on Juice	Average Starch in ppm				
			Temp. °C	Mixed Juice	Clear Juice	Raw Sugar	
						Unaff.	Affin.
15/1 to 17/1	Natural Enzyme	—	70 to 73	330	117	230	192
17/1 to 20/1	Added Enzyme	10	78 to 80	373	35	172	101
22/1 to 27/1	Added Enzyme	6	78 to 80	338	46	221	127
29/1 to 3/2	Natural Enzyme	—	70 to 73	312	61	212	155
5/2 to 10/2	Vacuum Flotation	—	—	297	66	171	113

Using a mean enzyme dosage of 7.2 ppm on mixed juice, starch destruction ranged between 78 and 93%, averaging about 86%. The sugar produced during a 24 hour period was consigned to Hulett's Refinery at Rossburgh for a filterability test run.

Though the results of this test were disappointing, most of the blame for the poor performance can be linked to the high insolubles and ash content of the sugar, brought about by poor clarification.

In the new season, sugar produced using enzyme treatment of clarified juice and syrup will be again evaluated in the Refinery. Following these trials a more realistic assessment of the refining qualities of sugar manufactured in a factory using bacterial enzymes to hydrolyse starch should be possible.

The Cost of the Enzyme Process

The costs of installations for an enzymatic hydrolysis process depend upon the point of application. If enzyme addition to the evaporators proves successful, the cost of an installation would be very small. Application in the mixed juice or clarified juice stage would necessitate certain installations, notably retention tanks.

Chemical costs depend upon the price of the enzyme. The costs estimated at Darnall and Melville were based on a tentative enzyme price of R1.45 per pound. At this price the cost of a process using 10 ppm on mixed juice is R0.34 per ton of sugar, plus the chemical costs of the defecation. At 6 ppm, enzyme costs drop to R0.21 per ton of sugar. If retention times can be increased, the amount of enzyme used can be reduced.

During the industrial trial at Darnall, the chemical costs totalled R0.33 per ton of sugar (enzyme R0.27, defecation R0.06). This figure compares favourably with the chemical costs of vacuum flotation.

Acknowledgements

The authors would like to thank the following for assistance given during the investigation.

The management and staff of Melville Sugar Estates and Hulett's factories, Amatikulu, Darnall and Mount Edgecombe, for making the necessary arrangements and giving their consent to the industrial experiments.

Mrs. E. M. J. Swart, Mrs. A. Atkinson, Mrs. R. E. Buchanan and Miss P. B. G. Stewart for their assistance in the laboratory investigations and during factory experiments at Melville.

The staff of Hulett's Research & Development laboratories, in particular Mr. I. A. Smith, for assistance in the tests at the Hulett factories.

Finally, particular thanks are due to the management of Chemical Services (Pty) Ltd., for supplying Bactamyl D-200 for the trial at Melville, for making available the technical assistance of Messrs. P. Morton and N. C. Gray, and for directing the interest of the industry back to bacterial enzymes.

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Discussion

Mr. Comrie: Does Mr. Jennings think it will be possible to remove the enzyme after it has attacked the starch and use it again?

Mr. Jennings: I do not think there is any method whereby the enzyme can be recovered.

Dr. Roth: The enzymes are inactivated after they have done their job.

Mr. Carter: In further investigations where bacterial enzymes are to be used, will an attempt first be made to use naturally occurring enzymes, bacterial enzymes only being added as required?

Mr. Jennings: This is not envisaged as the conditions are different. A two-stage process would be required where natural enzymes would be used first in a retention tank at the required temperature and pH and then the bacterial enzyme would have to be introduced.

Mr. Carter: We have four tanks available at Tongaat but are only using three.

The temperature we use for natural enzymes is about 75°C, approximately the same as used for bacterial enzymes. The pH varies a lot, 6.5 in your case and 5.7 in our case, but we do know that we could operate at the higher pH. I envisage removing 60% of the starch with natural enzymes and 30% with bacterial enzymes.

Mr. Jennings: Use of the bacterial enzyme means there is no loss of sucrose. However, I suggest we investigate the economic aspects of loss of sucrose against cost of bacterial enzymes by co-operating with you in an investigation at Tongaat.

Mr. Carter: With Rabe sugar you obtained a flow rate of 120 tons per filter which coincides with Yamane's work about starch being the bugbear of the industry. And yet in this paper with Empangeni sugar having a starch content of 550 ppm and a high ash content the throughput is 60 t.p.h. compared to a low starch sugar giving only 17 t.p.h. We appear to have been misled into thinking that starch is the main enemy.

Mr. Jennings: If all other things are equal then starch is one of the main causes of bad filtration in a refinery.

Mr. Cargill: Have the authors firmly decided that it is not practical to use the enzymes in the syrup stage?

Mr. Jennings: We have not decided yet although the reaction rate is slower in the syrup, probably due to viscosity. The Australians are thinking of adding the enzyme to the syrup and we will investigate further in the coming season.

Mr. Archibald: When comparing the vacuum flotation process and the enzyme process it must be remembered that the buyers of our sugar demand a certain specification, say 200 ppm starch. As much as possible is removed by the vacuum flotation process and this costs a certain amount whereas with

the enzymatic removal at a cost of say 30c per ton you may be reducing to as low as 100 ppm. It is easy to adjust the enzyme process by cutting down the amount of enzyme and bringing the starch level back to say 190 ppm with a consequent reduction in cost.

Mr. Alexander (in the chair): The buyers would quickly enough alter their specifications.

Dr. Matic: The processes are fundamentally different. Starch is removed physically in the Rabe process whereas in the enzymatic process the starch is decomposed.

The table of refinerabilities in the paper shows that starch is still the main trouble but we must also concentrate much more than we have been doing on good clarification in the factories.

In both Mauritius and Australia at present the emphasis is on efficient clarification.

Mr. Alexander: Hulett's Refinery intends to run a Mutual Clarification Control project in the coming season in conjunction with the Hulett sugar mills.

It is also to be noticed now that overseas importers and refiners are placing more emphasis on colour.

Mr. Gunn: We have today discussed two forms of starch removal, by flotation and by the use of enzymes. The Tongaat Sugar Company has patented a process which combines these methods. One half of the juice flow is scientifically subjected to attack by natural enzymes so that about 60% of the starch is degraded. The other half of the juice goes through a vacuum flotation plant at a high pH of 10.0 to 10.5 with a consequent removal of starch and a decrease in silica, calcium salts and magnesia. Not much polyelectrolyte is required to obtain good flotation. The juices are then combined and the high pH stream is neutralised by the low pH stream to a pH of 8.0. Automatic controllers are used for the proportioning.

Thereafter the normal clarification process is followed.

Up to 85% starch is removed, and ash is decreased by from 5% to up to 15%.

The cost, including the lime needed for defecation, is estimated at 18c per ton of sugar against the estimated 34c per ton for injected enzymes and 40c per ton for flotation.

The process has been patented throughout the sugar world but no charge will be made for its use by South African sugar factories.

Mr. Ashe: The Australians were not keen to discuss starch and were not keen to let one linger near their retention tanks. They are taking out the Bach and Rapi-Dorr clarifiers and replacing them with another clarifier supplied by Dorr-Oliver which they claim improves clarification.

Dr. Matic: The Australians are going in for hot liming either just before or just after the flash tank. Settling of the floc was not satisfactory so they had to increase the capacities of their clarifiers and use a considerable amount of flocculant. They claim a marked increase in juice quality as a result.

Mr. Gunn: Tongaat has been degrading starch by natural enzymes for about ten years. We were degrading about 30 to 40% of the starch. By radically altering the design of the retention tanks, without increasing retention time, we last year produced an excellent sugar.

Mr. Lenferna: It should be mentioned that last season Tongaat remelted up to 80% of the B sugars.

Mr. Jennings: In connection with the two Empanjeni sugars mentioned in the paper I should like to make the point that the method of determination of starch in South Africa depends upon the colour formed by amylose with iodine and assumes that the ratio of amylose to amylopectin remains fairly constant. It has been found elsewhere that there can be considerable variations in the amylose/amylopectin ratio in which case the figure for starch content would bear no relationship to the actual starch content of the sugar.

Regarding the Australians being reticent about starch removal this may be because they are at present endeavouring to patent a starch removal process in South Africa.

Mr. Prince: If the amylose fraction was 100% then the starch analysis would bear no relation to the true amount of starch present. All investigators of the amylose/amylopectin ratio have invariably found

that the amylose fraction falls between a range of 22 to 28% so a 100% amylose fraction does not seem to be a possibility.

Mr. Jennings: I quoted 100% merely as an example but I believe recent workers have found fractions much higher than 28%.

Mr. Ashe: The first experiments on starch removal by enzymes carried out in South Africa by Haddon were done at Umfolozi.

Mr. Alexander: Tate & Lyle have also patented a vacuum flotation process for refining, using a flocculant. It is interesting to note how many of these processes have been patented since Mr. Rabe set the ball rolling.

Dr. Matic: We have checked on work done in America on determining the amylose/amylopectin ratio to see if the method used was reliable. As the peak may shift during the measuring of colour depending on the ratio, we tried to develop a new method of starch determination using a specific enzyme.

We received a certain amount of enzyme from England and Mr. Bruijn found that it worked satisfactorily on pure starch. Unfortunately the method cannot be used with juice as the enzyme also attacks gums.