ENZYMATIC PRODUCTION OF GLUCOSE SYRUP FROM GRAINS AND ITS USE IN FERMENTATIONS[1]

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ABSTRACT

Starch in ground samples of whole corn, wheat, and sorghum was enzymatically converted to D-glucose in 90–95% yield with a combination of alpha-amylase from either barley malt or *Bacillus subtilis* and glucoamylase from *Aspergillus* species. Gas-liquid chromatography of the trimethylsilyl derivatives of the syrup showed that maltose and isomaltose comprised less than 10% of the total sugar. Syrups containing 20–25% D-glucose were produced in 24–48 hr. Approximately 20–25% of the original grain solids were recovered by filtration after enzymolysis. The nitrogen content of the recovered solids was about three times higher than the original grain. Some nitrogen was solubilized during enzymolysis and appeared in the syrups at a concentration of approximately 2 mg./ml. Two of the three strains studied, *A. awamori* NRRL 3112 and *A. niger* NRRL 3122, proved to be the most efficient in total starch-to-glucose conversion, and were low in transglucosidase activity. The syrups were employed for the production of several microbial polysaccharides, citric acid, fumaric acid, and 2-ketogluconic acid. Product yields equal to or surpassing those obtained with commercial D-glucose were realized.

The principal industrial use of glucoamylase is in the production of crystalline D-glucose. For this purpose crude preparations of the enzyme must be treated to remove transglucosidase which forms isomaltose and other oligosaccharides, since these sugars interfere in the crystallization process. Other industrial uses of glucoamylase can be envisioned in which the presence of relatively low amounts of isomaltose would not be detrimental. For example, glucose syrups produced by glucoamylase from crude starch sources, such as whole grains or grain flours, could be used as a carbohydrate source for a variety of fermentation processes. Moreover, in many fermentations it is desirable to remove the solids from the grain syrups before their use. Such solids may have potential commercial value as supplements to animal feed. It is the purpose of this paper to explore the feasibility of these syrups for industrial use and to determine the amount of isomaltose produced by unrefined glucoamylase preparations.

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Materials and Methods

Two strains of *Aspergillus niger,* namely NRRL 337 and NRRL 3122, and one strain of *A. awamori* NRRL 3112 were used for the production of glucoamylase. Stock cultures were maintained on yeast-malt agar slants (1) and transferred at 4-week intervals. The inoculum medium (750 ml.), containing 5% ground corn and 0.25% yeast extract, was placed in a Fernbach flask and inoculated with 50 ml. of a 48-hr. preinoculum mold culture. The mold was incubated for 36 hr. at 28°C. on a rotary shaker at 200 r.p.m. The inoculum (750 ml.) was added to a 20-liter stainless-steel nonbaffled fermenter containing 10 liters of sterile medium aerated through a pipe sparger. Two media were used for enzyme production. The first consisted of 15% whole ground No. 1 yellow corn and 2% corn steep liquor at a presterilization pH of 4.5. The inoculated medium was held for 96 hr. at 28°–30°C. Agitation was at 400 r.p.m. and air was admitted at the rate of 0.5 v./v./min. The second consisted of 20% whole ground corn slurry with pH adjusted to 4.5. The inoculated medium was held for 96 hr. at 35° C. Agitation was at 600 r.p.m. and aeration was 1.5 v./v./min. (2). The culture was filtered through Whatman No. 1 paper with the aid of suction and stored under toluene at 5°C. Loss of enzyme activity was negligible after 3 months. Glucoamylase activity was determined by the method described in Tech. Bull. 2-122 of Miles Laboratories (3). A glucoamylase unit (GU) is the amount of enzyme that will catalyze, under the assay conditions described, the production of 1 g. of glucose from starch in 1 hr. at 60°C.

The medium for production of alpha-amylase by *B. subtilis* NRRL B-941 consisted of 6% ground No. 1 yellow corn, 7% dry distillers' solubles, 0.1% K_2HPO_4, 0.02% $MgSO_4 \cdot 7H_2O$, and 0.001% each of $MnSO_4$, $Fe_2(SO_4)_3$, and NaCl at pH 6.5. Slants of freshly transferred stock cultures were used to inoculate preinoculum flasks containing 50 ml. of the medium in 300-ml. Erlenmeyer flasks. Cultures of *Bacillus subtilis* were incubated at 31°C. on a rotary shaker. After 1 to 2 days' growth, 5 ml. of the preinoculum was transferred to 50 ml. of inoculum medium. In 2 days the contents of the inoculum flasks were transferred to 300 ml. of medium in indented 2,800-ml. Fernbach flasks and allowed to incubate for 3 days. Cells and other solids were removed by centrifugation, and the pH was ajusted to 7.0. Negligible loss in activity for the bacterial amylase was noted after 4–6 weeks when it was stored at 5°C. Amylase production was measured as SKB units (4).

In a typical laboratory conversion, 200 g. as-is basis of whole ground cereal grain (e.g., corn — 63% starch), 2 g. barley malt (or 40 ml. bacterial alpha-amylase), and 500 ml. water were mixed in a Fernbach

flask. This mixture was heated in a boiling-water bath with constant agitation until the corn began to thicken at about 65°C. Heat was removed and the flask was shaken for 30 min. to liquefy the gelled corn, after which the contents were steamed for 20 min. to ensure complete gelatinization. The pH was adjusted to between 3.5 and 4.0 and the flask incubated at 60°C. on a shaker. Forty units of glucoamylase was added to completely convert the starch to D-glucose in 24 to 48 hr. The hydrolysate containing 22% D-glucose was easily filtered with the aid of suction. After filtration, 96% of the theoretical yield of D-glucose was recovered. D-Glucose recovery values were corrected from total reducing sugars by both glucose-oxidase and gas-chromatographic analysis of the filtrates.

For greater convenience in handling large numbers of grain or enzyme samples, smaller volumes were used. Five grams of ground grain, 0.1 g. barley malt containing 100 to 120 SKB units/g. of alpha-amylase (or 1 ml. bacterial alpha-amylase containing 20–25 SKB units/ml.) and 20 ml. water were mixed in 50-ml. Erlenmeyer flasks. The starch was gelatinized as described above. The pH was adjusted, and predetermined levels of glucoamylase were added. The flasks were incubated in a shaking water bath at 60°C. for 24–48 hr. The contents of the flasks were diluted to 200 ml. in a volumetric flask and filtered. Aliquots of the filtrate were analyzed for D-glucose, total reducing sugars, and soluble nitrogen. Residual solids were determined by weighing the dried residue on the filter papers. No significant differences between the two methods were observed.

For 10-liter conversions, 3 kg. corn was mixed with 30 g. barley malt and 7.5 liters water. Unbaffled 20-liter fermenters were used as converters and were equipped with large stirrer paddles to minimize clumping of the corn during heating. The gelatinization procedure was the same as that used for flasks. After cooling, the pH was adjusted and 600 units of glucoamylase added. The operating temperature of the fermenters was controlled with the available hot water supply, which ranged from 55° to 59°C.

Nitrogen was determined by a modification of the procedure described by Kanchukh (5) using Nessler's reagent (6). Reducing sugars were determined by the method of Somogyi (7) or with ferricyanide reagent (8). D-Glucose was estimated by the glucose-oxidase method of Hill and Kessler (9) modified by substituting tris-buffer (pH 7.6) for potassium phosphate.

Isomaltose in syrups was determined by gas-liquid chromatography. Aluminum columns, 4 ft. long and 1/4 in. o.d., were filled with silanized Chromosorb W (HMDS), coated with 3% silicone rubber (SE-52) and

heat-cured before use. Helium was the carrier gas (50 ml./min.), the thermal conductivity detector temperature was 280°C., injection port temperature was 300°C., and cell current was 150 ma. Trimethylsilyl derivatives of sugars were prepared by the method of Sweeley *et al.* (10). Chromatograms were obtained in 15 min. by programming from 155° to 275°C. at 20°/min., starting 1 min. after injection of 40 to 50 microliters of 1% sample.

Results and Discussion

Liquefaction of gelatinized starch by enzymes present in barley malt, or *Bacillus subtilis* culture filtrate (20–25 SKB units/ml.), provided the necessary thinning before addition of glucoamylase. Further thinning was accomplished with a culture filtrate from one of the three Aspergilli. Shown in Table I are the amounts of alpha-amylase

TABLE I

STARCH LIQUEFACTION (ALPHA-AMYLASE ACTIVITY) BY CULTURE FILTRATES OF GLUCOAMYLASE-PRODUCING ASPERGILLI[a]

TIME	NRRL 337 (48 SKB units/ml.)	NRRL 3112 (16 SKB units/ml.)	NRRL 3122 (1 SKB unit/ml.)
min.	*cp.*	*cp.*	*cp.*
0	3,300	2,900	3,100
5	240	1,000	2,800
10	90	380	2,700
60	90	120	920
120	85	87	180
180	77	87	125

[a] 20 g. corn flour + 180 ml. water; hold at 75°C. for 15 min., cool to 60°, add 2 ml. filtrate.

found in these filtrates and their efficacy for starch liquefaction. It can be seen that the rate of starch liquefaction is related to the amount of

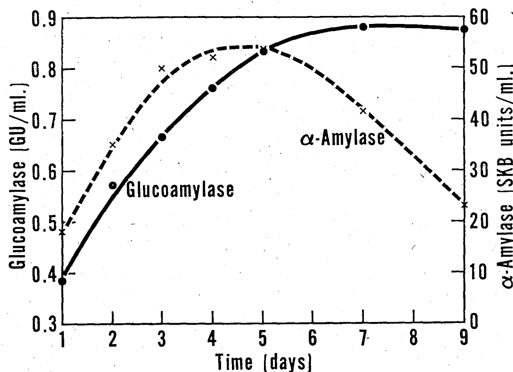

Fig. 1. Production of enzymes by *Aspergillus niger* NRRL 337.

enzyme added. Production of alpha-amylase by NRRL 337 is shown in Fig. 1. Both glucoamylase and alpha-amylase activities in the culture filtrate rapidly increase during the initial 4–5 days' incubation. Thereafter, the alpha-amylase activity diminishes.

When culture filtrates containing glucoamylase were added to highly concentrated slurries of cereal grains or flours (30%+), the concomitant presence of alpha-amylase greatly speeded the initial rate of conversion by rapidly thinning the almost solid gelatinized masses.

As shown in Table II, the yield of glucoamylase is markedly affected by the medium and physical environment. It can be seen that the medium containing 20% corn, in conjunction with increased aeration and temperature, gave higher amounts of glucoamylase. Strains NRRL 3112 and 3122 produced consistently higher glucoamylase activities than strain 337. No attempts were made to run 15% corn plus 2% corn steep liquor at the higher aeration, agitation, and temperature used in the high-potency medium. Shaker flask experiments invariably showed that substitution of part of the corn with other nitrogen sources resulted in decreased yields.

TABLE II

GLUCOAMYLASE PRODUCTION BY ASPERGILLI IN 20-LITER FERMENTERS

NRRL No.	Low Potency [a]		High Potency [b]	
	GU/ml.	*final pH*	*GU/ml.*	*final pH*
337	0.9	4.05	2.8	3.30
3112	2.3	3.95	11.4	2.90
3122	3.6	3.30	10.8	2.90

[a] Conditions: 15% corn + 2% corn steep liquor; 400 r.p.m.; 30°C.; 0.5 vol. air.
[b] Conditions: 20% corn; 600 r.p.m.; 35°C.; 1.5 vol. air.

The formation of transglycosidase by molds grown on high- and low-potency media was investigated. Table III shows that somewhat

TABLE III

ISOMALTOSE PRODUCTION BY LOW- AND HIGH-POTENCY CULTURE FILTRATES OF ASPERGILLI

NRRL No.	Filtrate [a]		Isomaltose	Isomaltose/GU [b]
	GU/ml.	*ml.*	*% of total sugar*	*% of total sugar*
337	0.8	1.25	15.2	15.2
337	1.5 [c]	1.25	16.4	9.0
3112	2.6	0.40	1.54	1.48
3112	10.8 [c]	0.40	2.65	0.61
3122	3.7	0.30	0.36	0.32
3122	12.6 [c]	0.30	1.47	0.39

[a] Specified quantity of filtrate added to 16 ml. of 25% maltose, 0.1*M* acetate buffer (pH 5.0), incubated at 60°C. for 24 hr.
[b] Calculation: % isomaltose/GU = (% total sugar)/(GU/ml. × ml. filtrate).
[c] High-potency filtrate.

less transglycosidase was produced by NRRL 3112 and 3122 on a volume basis. On a GU basis, less transglycosidase was produced with the high-potency filtrates of NRRL 337 and 3112, whereas with 3122, glucoamylase and transglycosidase were produced in proportionate amounts. It is especially noteworthy that both NRRL 3112 and 3122 produced considerably less transglycosidase per GU formed than did 337. The isomaltose values shown in Table III are maximal, since the pH was set at 5.0 to more nearly approach optimum conditions for transglycosidase activity. Figure 2 shows the conversion of starch

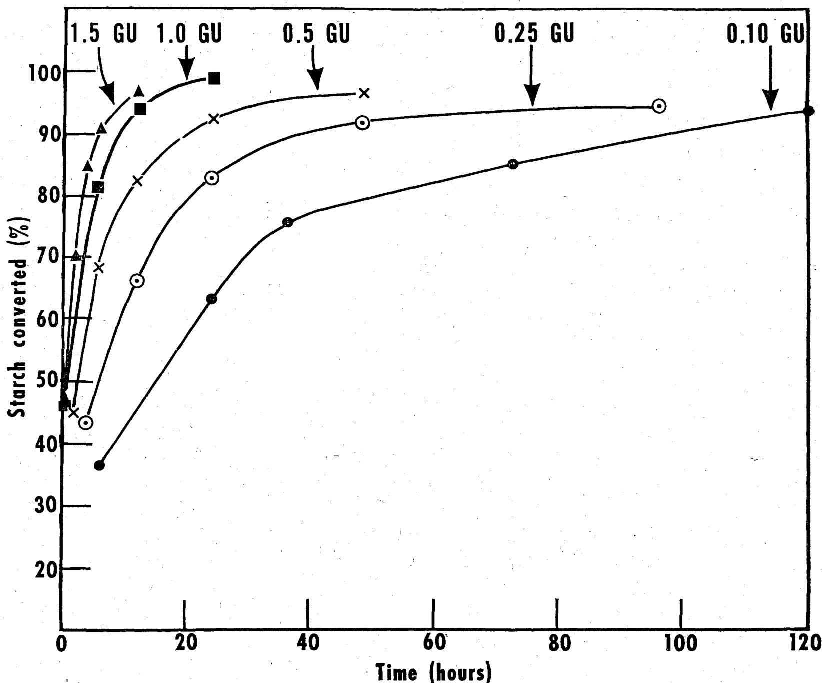

Fig. 2. Conversion of starch at 60°C. in 5 g. corn to D-glucose by varying amounts of NRRL 3112 glucoamylase.

to D-glucose as catalyzed by the addition of NRRL 3112 glucoamylase. Various amounts of enzyme were added to 5-g. samples of corn. In each case nearly theoretical conversions were obtained, the rates being proportional to the amount of enzyme added. One-half to 1.0 GU converted the starch from 5 g. of corn to D-glucose in 24 hr. at 60°C. Rates were slightly lower at 55°C., and at 65°C. the enzyme was slowly inactivated. The pH of this conversion was 3.8. Rates changed little between pH 3.2 and 4.5, but decreased sharply outside this range.

Our use of 0.1*M* acetate buffer for pH control of the enzymolysis reaction mixture was shown later to be superfluous, since the pH increased only 0.2–0.3 unit when the buffer was omitted and glucose yields were not affected. Similar results were obtained with NRRL 337 glucoamylase.

The presence of transglycosidase activity in culture filtrates of NRRL 337, 3112, and 3122 led to the production of unfermentable carbohydrates, notably isomaltose and panose, during the enzymolysis of starch. Consequently, less than quantitative yields of D-glucose were obtained. Paper chromatography, however, indicated significant panose formation only when maltose was substituted for starch as a substrate. Isomaltose was the primary product of transglycosidase activity on starch.

An attempt was made to minimize transglycosidase activity by carrying out the conversion of starch to glucose at low pH. Reaction mixtures containing NRRL 337 and 3112 culture filtrates were adjusted to various pH levels. Figure 3 shows the results of gas-chromatographic

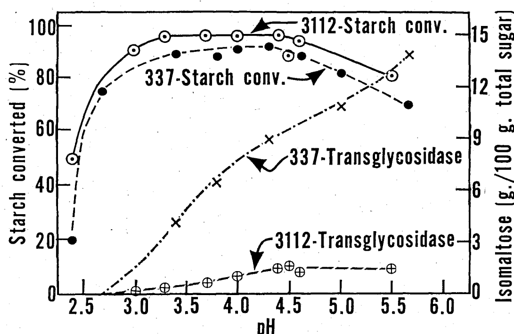

Fig. 3. Effect of pH on transglycosidase activity and starch to D-glucose conversion by *A. niger* NRRL 337 and *A. awamori* NRRL 3112.

analysis of the sugars produced after incubation for 24 hr. at 60°C. It can be seen that the reaction mixtures gave very little isomaltose at lower pH but produced significantly more as the pH increased. The pH values necessary for maximum conversion of starch to glucose were higher than the pH values at which transglycosidase was inactivated. However, isomaltose production by NRRL 3112 filtrates was low, even at higher pH.

Figure 4 shows a typical gas-liquid chromatogram of sugars present in an enzymatically converted sorghum slurry. Filtrates from the three strains of *Aspergillus* yielded syrups with 15% sugar. Peaks shown for alpha- and beta-glucose were attenuated electronically eightfold over

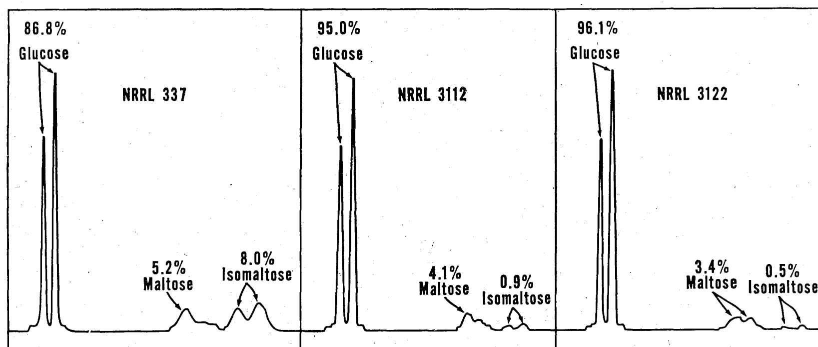

Fig. 4. Gas-liquid chromatograms of trimethylsilyl ether derivatives of sugars present in syrups produced by the enzymolysis of ground sorghum slurries by *Aspergillus* culture filtrates.

those of the disaccharides. Without this variation in sensitivity, estimation of maltose and isomaltose with the integrator on the recorder would not have been possible. The enzymes of strain NRRL 337 produced 9 and 16 times as much isomaltose as those of NRRL 3112 and 3122 respectively. The percentage of isomaltose was somewhat higher in the more concentrated glucose syrups (11). Continued incubation beyond maximal glucose production also resulted in increased formation of isomaltose. Depending upon pH, glucose concentration, and duration of incubation, enzymes from culture filtrates of NRRL 3112 and 3122 catalyzed the production of 0.2 to 2.0% isomaltose in glucose syrups, whereas NRRL 337 enzymes catalyzed the formation of 5 to 10% isomaltose. Gas chromatographic analysis showed the presence of disaccharide sugars with the same retention time as maltose. Paper chromatography confirmed the presence of maltose.

Table IV shows the conversion of starch in ground whole wheat to glucose as catalyzed by glucoamylases from the three strains of *Aspergillus*. Higher glucose yields were obtained in the presence of NRRL

TABLE IV
CONVERSION OF GROUND WHOLE WHEAT WITH GLUCOAMYLASE PRODUCED BY ASPERGILLI [a]

NRRL No.	Starch Converted	Sugars Found by GLC [b]		
		Glucose	Maltose	Isomaltose
	%	*% of total*	*% of total*	*% of total*
337	90.5	90.4	2.9	6.6
3112	94.7	99.0	0.6	0.5
3122	96.2	98.9	0.6	0.5

[a] 0.5 GU/5 g. wheat; reaction ran 72 hr. at 60°C., pH 4.0.
[b] Gas-liquid chromatography of trimethylsilyl ether derivatives.

3112 and 3122 culture filtrates. The amount of disaccharide produced by NRRL 337 greatly exceeds that produced by 3112 and 3122.

The carbon-nitrogen balance during enzymatic conversion of starch in cereal grains is shown in Table V. All values were calculated on a

TABLE V
CARBON-NITROGEN BALANCE OF THE ENZYMATIC CONVERSION OF STARCH IN GRAINS TO D-GLUCOSE WITH *A. awamori* NRRL 3112[a]

	CORN	WHEAT	SORGHUM
	g.	g.	g.
Original grain before enzymolysis	100	100	100
Starch	71.7	65.4	69.2
Nitrogen	1.50	2.59	1.50
Original grain after enzymolysis			
D-Glucose	74.8	68.6	71.6
Soluble nitrogen	0.34	1.07	0.38
Residual solids	22.7	22.7	24.6
Nitrogen residue	1.19	1.42	1.24

[a] 20 GU/100 g. grain; reaction time was 30 hr. at 60°C., pH 4.0.

dry basis. At least 95% of the starch of all three grains was converted to glucose in 30 hr. with a culture filtrate from strain NRRL 3112. Wheat, before hydrolysis, contained nearly twice as much nitrogen as the other grains. A larger amount of nitrogen was also solubilized from wheat: 41 *vs.* 25%. Nearly 25% of the original weights of the grains appear as residual solids. The nitrogen in the residual solids increased about threefold over that in the original grains, making residual solids a potential protein concentrate for animal feeds. The amounts of nitrogen solubilized by culture filtrates from strains NRRL 337 and 3122 were also determined. Results show both strains to be similar to NRRL 3112.

Glucose syrups produced by enzymatic hydrolysis of various cereal grains and flours were substituted for crystalline glucose in several different fermentations. Table VI shows the effect of the glucose syrups on the production of citric and 2-ketogluconic acids by fermentation. The results show that glucose syrups made from sorghum, corn, or corn flour can satisfactorily substitute for both crystalline glucose and inorganic nitrogen in the production of these acids. The low yields obtained with wheat syrup were likely due to the high concentration of soluble nitrogen in this product. This view is strengthened by the fact that additional nitrogen added to the wheat syrup medium depressed acid yields still further. It has been shown that citric acid yield is affected adversely by excessive amounts of inorganic nitrogen or by corn steep liquor (12). Unpublished work from our laboratory has shown that the yield of 2-ketogluconic acid is reduced drastically by

TABLE VI
UTILIZATION OF GLUCOSE SYRUPS FOR THE PRODUCTION OF CITRIC ACID AND 2-KETOGLUCONIC ACID

CARBOHYDRATE SOURCE	CITRIC ACID [a]	2-KETOGLUCONIC ACID [b]
10 g. D-glucose	*g.*	*g.*
Dextrose	5.9 [c]	9.4 [d]
Corn	6.1 [e]	9.1 [e]
Wheat	2.4 [e]	1.9 [e]
Sorghum	5.8 [e]	9.1 [e]
Corn flour	5.9 [e]	9.3 [e]

[a] By fermentation with *A. niger* NRRL 372.
[b] By fermentation with *Serratia marcescens* NRRL B-486.
[c] Medium described by Moyer (12).
[d] Medium described by Misenheimer *et al.* (13).
[e] Grain syrup was substituted for both dextrose and nitrogen described in the published medium.

excessive nitrogen in the medium.

Glucose syrup from corn was also tested as a carbon and nitrogen source in the production of fumaric acid as described by Rhodes *et al.* (14). The syrup served as both carbohydrate and nitrogen source; fumaric acid yields (4.8 g./100 ml.) exceeded the values obtained by the published method (3.8 g./100 ml.).

The effect of glucose syrups from cereal grains on the production of polysaccharide B-1973 (15) is shown in Table VII. Significantly higher

TABLE VII
EFFECT OF GRAIN SYRUPS ON POLYMER PRODUCTION BY *Arthrobacter viscosus* NRRL B-1973

CARBOHYDRATE SOURCE	POLYMER PRODUCED WITH ENZYME-HYDROLYZED CASEIN (EHC)		
	0.30% EHC	0.15% EHC	No EHC
3 g. D-glucose	*g.*	*g.*	*g.*
Dextrose	1.07		
Corn	1.33	1.15	0.73
Wheat	1.55	1.52	1.58
Sorghum	1.43	1.35	0.80

yields of polymer were obtained when wheat syrup was substituted for crystalline glucose in the medium. Enzyme-hydrolyzed casein was not required when wheat syrups were used. Syrups made from corn or sorghum required supplementation with a small amount of enzyme-hydrolyzed casein to realize yields equivalent to those obtained with media employing crystalline glucose as a carbon source.

Similar results were obtained with polysaccharide produced by *Xanthomonas campestris* NRRL B-1459 (16). Yields and viscosities were generally increased in culture liquors of the organism grown on grain syrups. Production at 5% carbohydrate levels resulted in viscosities as high as 10,000 to 12,000 cp. with a comparable increase of

isolated product; i.e., an improvement in the yield of polymer from batch-type fermentations. With wheat syrup, yields increased as the concentration of distillers' solubles was decreased. However, yields dropped sharply with all syrups when distillers' solubles were omitted completely from the medium.

Glucose syrup from corn was also tested in polysaccharide production with *Cryptococcus laurentii* var. *flavescens* NRRL Y-1401 (17) and with *Hansenula holstii* NRRL Y-2448 (18). The yields were as high as the D-glucose control in both cases, although some discoloration of the polymers was observed.

Owing to the variability of nitrogen and carbohydrate requirements in different fermentations, some cereal grains would be more applicable in a particular process than would others. Should a syrup of high nitrogen content be desired, wheat would be preferable. Substrates containing less nitrogen can be obtained from corn, corn flour, or sorghum.

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