557. *Production of Mannitol by a* Lactobacillus *causing Ropiness in Cider.*

By S. A. Barker, E. J. Bourne, Elisabeth Salt, and M. Stacey.

The major change in carbohydrate content effected during growth of a ropy-cider organism on a medium containing apple juice and yeast extract is production of mannitol from the fructose present in the apple juice. The nature of the " ropy " precipitate is discussed.

Ropy cider is cider which has become highly viscous and when poured from a bottle forms a wide continuous stream. This disorder is generally accompanied by turbidity and deposition of a " ropy " precipitate. One of the most common producers of ropiness in cider was isolated by Millis [1] and shown to be a Gram-positive *Lactobacillus* which was

[1] Millis, Ph.D. Thesis, Bristol, 1951.

related to, but not identical with, *L. brevis*, *L. buchneri*, and *L. pastorianus*. The object of the present investigation was to determine the changes brought about by this *Lactobacillus* in a medium containing apple juice and yeast extract, with particular reference to the carbohydrate constituents.

Fractionation of sugars, oligosaccharides, and smaller molecules present in ropy apple juice was effected on a charcoal column.[2] The major component isolated from the aqueous eluate was crystalline mannitol which was characterised by its infrared spectrum and its rotation in borate solution and as its hexa-acetate. The glucose and fructose detected in the mother-liquors were characterised as their *O-iso*propylidene derivatives. Galacturonic acid and amino-acids were also found in the aqueous eluate. From the fractions eluted with aqueous ethanol crystalline sucrose and two trisaccharides were obtained. Examination of the sugars present in the original medium indicated that the major change effected by the ropy-cider organism was the production of mannitol. Quantitative determination of the changes occurring (Table 1) confirmed this and showed the gradual disappearance of glucose, fructose, and amino-acids.

TABLE 1. *Changes effected by the ropy-cider organism.*

Time of growth (days)	pH	% w/v pptd. by 2 vols. of EtOH	Mannitol (%) isolated (corr.)	Total reducing substances (%)	Fructose (%)	Glucose (%)	Amino-acids (%) (corr.)
0	4·96	0·28	—	7·57	5·0	2·57	0·717
1	4·75	0·31	—	6·85	—	—	—
2	—	0·27	0·74	6·90	4·79	2·11	—
3	4·30	0·26	2·14	4·85	3·70	1·15	0·757
4	—	0·28	2·84	3·24	—	—	0·600
6	—	0·29	3·76	2·82	—	—	—
8	—	0·25	3·92	1·78	1·50	0·28	0·432
9	—	0·32	4·57	1·39	1·15	0·24	—
11	4·08	0·23	5·00	—	—	—	—
16	4·25	0·28	4·51	0·84	—	—	—
20	—	0·25	4·96	0·79	0·72	0·07	0·614
32	4·00	0·27	4·87	0·19	—	—	0·311

The soluble polysaccharide fraction ($[\alpha]_D$ +48·8°) in ropy apple juice was isolated by dialysis, fractionation with " Cetavlon " (cetyltrimethylammonium bromide),[3] to remove nucleic acid, and removal of proteins by Sevag, Lackman, and Smolens's method.[4] On hydrolysis it gave mannose, glucose, galactose, and arabinose. This polysaccharide fraction was separated into a mannan, $[\alpha]_D$ +75·6° (isolated *via* its copper complex), and a polysaccharide, $[\alpha]_D$ +18·0°, incorporating glucose, galactose, and arabinose. A mannan with $[\alpha]_D$ +76·2° was present in the original yeast extract. A polysaccharide fraction isolated from the original apple juice and also incorporating glucose, galactose, and arabinose showed a higher specific rotation (+60·0°) than that present in ropy apple juice. The amount of material which was isolated by addition of 2 volumes of ethanol to cultures of ropy cider grown for various times (Table 1) and would contain the soluble polysaccharides remained almost constant.

Analysis of the ropy precipitate after extraction with sodium cholate [5] indicated that it consisted of protein, tannin (cf. Williams [6]), nucleic acid, and a polysaccharide fraction containing mannose and glucose units.

The ropy-cider organism was grown on synthetic media containing glucose or fructose, ascorbic acid, and yeast extract in mineral medium. The results suggested that, while glucose speeds the initial growth of the organism, fructose is needed for the formation of both the characteristic ropy mucilage and mannitol.

[2] Whistler and Durso, *J. Amer. Chem. Soc.*, 1950, **72**, 677.
[3] Dutta, Jones, and Stacey, *Biochim. Biophys. Acta*, 1953, **10**, 607, 613.
[4] Sevag, Lackman, and Smolens, *J. Biol. Chem.*, 1938, **124**, 425.
[5] Henry and Stacey, *Nature*, 1943, **151**, 671.
[6] Williams, Ann. Rept. Agr. Hort. Res. Sta. Long Ashton, Bristol, 1952, p. 219.

Experimental

Preparation of Ropy Apple Juice.—Depectinised Bramley's Seedling apple juice containing 1% of "Difco" yeast extract was passed through a Seitz filter, adjusted to pH 4·8, and sterilised in an autoclave for 15 min. at 7—10 lb. per sq. in. The sterile medium was inoculated with the ropy-cider organism and incubated for 11—14 days at 30°/4 cm. in a vacuum-desiccator over 25% aqueous sodium hydroxide and pyrogallol.

Fractionation of Ropy Apple Juice.—(a) *Sugars.* The ropy medium (400 c.c.) was centrifuged, neutralised, and fractionated [2] on a charcoal column (21 × 3·8 cm.). Paper chromatography and ionophoresis [7] of the aqueous eluate (1600 c.c.) showed the presence of, *inter alia*, glucose, fructose, galacturonic acid, and amino-acids. The freeze-dried eluate (51·15 g.) gave mannitol (14·05 g.), m. p. and mixed m. p. 164—166·5°, $[\alpha]_D^{17}$ +28·7° (*c* 0·627 in aqueous borate) (confirmed by infrared analysis) (Found: C, 40·0; H, 8·0. Calc. for $C_6H_{14}O_6$: C, 39·5; H, 7·75%). Acetic anhydride and zinc chloride gave mannitol hexa-acetate, m. p. and mixed m. p. 123·5—125° (Found: C, 49·6; H, 6·0. Calc. for $C_{18}H_{26}O_{12}$: C, 49·7; H, 5·9%). A portion (5·29 g.) of the residual elute, absorbed on kieselguhr (10 g.), was shaken with acetone (98·8 c.c.) and concentrated sulphuric acid (5·2 c.c.) for 6 hr. Partial hydrolysis (Bell [8]) gave a product (1·07 g.), m. p. 91·5—93·5° undepressed on admixture with 2 : 3-4 : 5-di-*O*-*iso*-propylidene D-fructopyranose and showing $[\alpha]_D^{18\cdot5}$ −24·9° (*c* 0·802 in $CHCl_3$) (Found: C, 55·7; H, 7·6. Calc. for $C_{12}H_{20}O_6$: C, 55·35; H, 7·75%). A second product (0·07 g.) had m. p. 150—152° undepressed on admixutre with 1 : 2-*O*-*iso*propylidene-D-glucose (Found: C, 48·55; H, 7·1. Calc. for $C_9H_{16}O_6$: C, 49·1; H, 7·3%).

Further elution with aqueous ethanol (2·5%, 500 c.c.; and 5%, 2·2 l.) gave a fraction (2·418 g.) containing amino-acids and a component identical in its paper chromatographic behaviour with sucrose. The latter crystallised from methanol and had m. p. and mixed m. p. 168—170°, $[\alpha]_D^{17}$ +65·6° (*c* 0·87 in H_2O) (Found: C, 42·1; H, 6·45. Calc. for $C_{12}H_{22}O_{11}$: C, 42·1; H, 6·5%).

Elution with 9 : 1 water–ethanol (1·7 l.) gave a fraction (0·371 g., $[\alpha]_D^{17}$ +28·8° in H_2O) which contained a single component with the mobility of a trisaccharide.[9] Acid hydrolysis gave glucose and fructose (identified paper-chromatographically). Another fraction (0·239 g.) eluted with 85 : 15% water–ethanol (1 l.) had $[\alpha]_D^{17}$ +11·8° in H_2O and contained two similar trisaccharides.

(b) *Polysaccharides.* Ropy apple juice (2640 c.c.) was dialysed against running water for 7 days. The insoluble material (2·224 g.) was removed by centrifugation and the soluble material (5·372 g.) recovered by concentration and freeze-drying. The latter material was redissolved in water and "Cetavlon" (cetyltrimethylammonium bromide) added until there was no further precipitation. The supernatant solution was then submitted to 22 Sevag separations [4] which removed most of the protein. The polysaccharide fraction (2·360 g.) remaining in solution had $[\alpha]_D^{17}$ +48·8° (*c* 0·431 in H_2O). Paper-chromatographic examination of its hydrolysate (2N-sulphuric acid at 100° for 4 hr.) showed the presence of mannose, glucose, galactose, and arabinose. Part (0·595 g.) of this polysaccharide was refractionated. On addition of alkaline Fehling's solution some of the polysaccharide (0·161 g.) was precipitated as a copper complex. The free polysaccharide had $[\alpha]_D^{18}$ +75·6° (*c* 0·40 in H_2O) and examination of its hydrolysate (as above) showed that it contained only mannose with a trace of galactose. The polysaccharide (0·035 g.) recovered from the supernatant solution had $[\alpha]_D^{18}$ +18·0° (*c* 0·54 in H_2O) and chromatography of its hydrolysate indicated the presence of glucose, galactose, and arabinose with a trace of mannose.

(c) *Insoluble ropy material.* The insoluble material, collected from ropy apple juice (900 c.c.) which had been incubated for 7 days, was thoroughly washed with water and then suspended in 2% aqueous sodium cholate [5] (38 c.c.). This suspension was added dropwise during 1 hr. to a further quantity of 2% aqueous sodium cholate (50 c.c.) at 60°. After cooling, the insoluble fraction A (0·444 g.) was recovered and the supernatant solution dialysed to remove sodium cholate. Further insoluble material B (0·323 g.) which separated was collected at the centrifuge. Addition of "Cetavlon" to the supernatant liquid gave fraction C (0·104 g.). A further fraction D (0·015 g.) was recovered by addition of two volumes of ethanol.

[7] Foster, *J.*, 1953, 982.
[8] Bell, *J.*, 1947, 1461.
[9] Barker and Carrington, *J.*, 1953, 3588.

Fractions A, B, C, and D were each hydrolysed (as above) and the hydrolysates analysed by paper chromatography and ionophoresis. Glycerol was found in A before hydrolysis. The hydrolysate of A contained glucose, glycerol, at least five amino-acids (one of which was arginine), and several unidentified components one of which could be detected by fluorescence in ultraviolet light and developed a yellow colour with ammonia: this component was believed to be chlorogenic acid. Fraction B contained no glycerol and after hydrolysis produced glucose, at least six amino-acids, and a component believed to be chlorogenic acid. The hydrolysate of fraction C contained ribose, glucose, glycerol, and amino-acids. The major component of fraction C was nucleic acid since it showed an ultraviolet absorption peak at 258 mμ, contained phosphorus, and on alkaline hydrolysis [10] gave cytidylic, adenylic, guanylic, and uridylic acid. Fraction D showed no ultraviolet absorption and the hydrolysate contained mannose and glucose.

Examination of Original Apple Juice.—Examination of depectinised Bramley's Seedling apple juice by paper chromatography and ionophoresis showed the presence of glucose, fructose, and small amounts of aspartic acid, glutamic acid, and neutral amino-acids.

Apple juice (500 c.c.) was dialysed against running water for six days; the soluble polysaccharide (0·311 g.), isolated as above, had $[\alpha]_D^{18}$ +60·0° (*c* 0·20 in H_2O). Analysis of the hydrolysate showed the presence of glucose, galactose, and arabinose.

Examination of Difco Yeast Extract.—No reducing sugars were detected in the yeast extract but spraying paper chromatograms with silver nitrate [11] disclosed a component with an R_F value similar to that of trehalose. Large amounts of amino-acids including aspartic acid, glutamic acid, arginine, and lysine were detected in the yeast extract.

1% Difco yeast extract (490 c.c.), treated as above, gave a soluble polysaccharide fraction (1·48 g.), $[\alpha]_D^{18}$ +27·8° (*c*, 0·54 in H_2O), which gave mannose together with a trace of glucose on acid hydrolysis. Formation of the copper complex and purification involving extraction with trichloroacetic acid [12] gave a mannan, $[\alpha]_D^{18\cdot5}$ +76·2° (*c* 1·3 in H_2O).

Quantitative Determination of the Changes occurring in Ropy Apple Juice.—Thirty-eight graduated tubes each containing the standard apple juice–yeast extract medium (20 c.c.) were sterilised and inoculated. At intervals after incubation at 30° some of the cultures were centrifuged and filtered through a No. 4 sintered-glass filter. The pH of each solution was measured and then a known volume mixed with two volume of ethanol to precipitate any soluble polysaccharide. In selected cases, the aqueous-alcoholic solutions remaining after precipitation of the polysaccharide were concentrated to a small volume to remove alcohol and then passed down a column (30 × 1 cm.) of Amberlite IRA-400 (OH^-). The mannitol was eluted with carbon dioxide-free water (300 c.c.; flow rate 5 c.c./min.), and the eluate neutralised, concentrated to a syrup, and extracted with methanol. Mannitol crystallised from the methanol extract when present and the eluate had zero optical rotation in all cases. When the mannitol was recovered from two solutions of mannitol (3·88%), fructose (6·69%), glucose (2·41%), sucrose (0·70%), and tannic acid (0·79%) by this standard procedure the recoveries were 87·0% and 90·7% respectively. The total reducing substances in the concentrated aqueous alcoholic solutions remaining after precipitation of the polysaccharide were determined by the Shaffer–Hartmann method.[13] Fructose was determined by the van der Plank method,[9] and the difference between the value for total reducing sugars and fructose was assumed to be due to glucose. For the determination of amino-acids the solution was passed down a column of Zeo-Karb 215 (H^+) which was washed with water until zero optical rotation indicated the complete removal of sugars. The column was then eluted with 3N-ammonia until the effluent gave no test for amino-acids with ninhydrin.

A standard solution (10 c.c.) containing arginine (0·073 g.), glycine (0·080 g.), glutamic acid (0·074 g.), fructose (0·261 g.), glucose (0·178 g.), and mannitol (0·254 g.) was separated in this way and gave an 87% recovery of amino-acids (0·197 g.). The results of the experiments are given in Table 1.

Growth of Ropy-cider Organism in Synthetic Media.—A medium was prepared containing fructose (9·06%), ascorbic acid (0·29%), Difco yeast extract (0·92%), magnesium sulphate (0·2%), sodium nitrate (0·2%), and ammonium sulphate (0·2%). A similar medium in which

[10] Davidson and Smellie, *J.*, 1952, 594.
[11] Trevelyan, Proctor, and Harrison, *Nature*, 1950, **166**, 444.
[12] Hiller and van Slyke, *J. Biol. Chem.*, 1922, **53**, 253.
[13] Shaffer and Hartmann, *ibid.*, 1921, **45**, 365.

glucose (9·11%) replaced the fructose was also prepared and after sterilisation both were inoculated with the ropy-cider organism and incubated (as above). Growth in fructose medium was slow and of a characteristic ropy nature, whereas in the glucose medium growth was fast and the precipitate powdery. The results of analyses are given in Table 2. Hydrolysis

TABLE 2. *Growth in synthetic media.*

Inoculation (days)	pH	Mannitol (%) (corr.)	Total reducing substances (%)	% (w/v) pptd. with 2 vols. of EtOH	Inoculation (days)	pH	Mannitol (%) (corr.)	Total reducing substances (%)	% (w/v pptd. with 2 vols. of EtOH
Glucose medium					*Fructose medium*				
0	4·96	None	9·17	0·223	0	5·00	None	9·05	0·247
5	—	,,	6·75	0·278	5	—	0·004	8·00	0·233
12	3·75	,,	7·55	0·233	12	3·65	3·55	3·76	0·303

of the polysaccharide fraction obtained from a fructose culture gave mannose, glucose, and galactose. Paper chromatography of a fructose medium indicated the presence of glycerol and erythritol as well as mannitol and the original fructose.

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CHEMISTRY DEPARTMENT, THE UNIVERSITY, BIRMINGHAM, 15.
CHEMISTRY DEPARTMENT, ROYAL HOLLOWAY COLLEGE,
ENGLEFIELD GREEN, SURREY.

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