APPLIED MICROBIOLOGY, March, 1965
Vol. 13, No. 2

Printed in U.S.A.

Fermentative Production of Exocellular Glucans by Fleshy Fungi

E. N. DAVIS, R. A. RHODES, AND H. RUSSELL SHULKE

Northern Regional Research Laboratory, U.S. Department of Agriculture, Peoria, Illinois

Received for publication 25 November 1964

ABSTRACT

DAVIS, E. N. (Northern Regional Research Laboratory, Peoria, Ill.), R. A. RHODES, AND H. RUSSELL SHULKE. Fermentative production of exocellular glucans by fleshy fungi. Appl. Microbiol. **13**:267-271. 1965.—Two specimens of higher fungi produced exocellular β-1,3-glucans when their mycelial forms were cultivated under submerged aerobic conditions. *Plectania occidentalis* NRRL 3137 consumed up to 6% glucose or xylose with about 30% conversion to polymer in a medium composed of hydrolyzed soy protein, salts, and thiamine. A 5% inoculum was used in a 10-day shaken fermentation. After dilution of the culture liquors and partial disruption of mycelia with a blender, solids were removed by centrifugation, and the polymer was precipitated by the admixture of 2 volumes of ethyl alcohol. A second polymer was formed in 40 to 65% yield by fermentation with *Helotium* sp. NRRL 3129, which in the imperfect stage would be identified as *Monilia* sp. It consumed up to 4% glucose, fructose, mannose, or sucrose in 60 to 72 hr. A 2% inoculum in a medium composed of commercial defatted soy flakes, phosphate, and thiamine in tap water gave a satisfactory fermentation. This polymer was precipitated by the addition of 0.5 volume of ethyl alcohol. Both organisms have a broad *p*H optimum on the slightly acidic side and did best at about 25 C.

In the course of an investigation of the products formed by submerged aerobic culture of mycelial forms derived from the spores or tissue of fleshy fungi, 488 specimens were collected locally and classified in 58 taxonomic groups. The isolates were cultured as vegetative growth on agar slants. Submerged fermentations by these organisms in a liquid soy protein-glucose medium were conducted in shaken Fernbach flasks, and the culture liquors were assayed for the conversion of glucose to products.

After several days of growth, fermentation liquors of two mycelial forms propagated from spores released from ripe asci of ascomycetes of the order Pezizales showed high viscosity, which indicated polymer formation. Laboratory-scale fermentations in shaken flasks were made to obtain information on the usual variables which control microbial product formation in aerobic fermentations. Because these products yield primarily glucose on acid hydrolysis and are at least partially hydrolyzed by the specific β-1,3-glucanases of *Rhizopus arrhizus* and a basidiomycete species described by Reese and Mandels (1959), both may be assumed to have the same primary structure. The presence of β-1,3 bonds places these polymers in the same general classification as a laminarin extractable from the brown seaweed, *Laminaria clonstoni* (Nelson et al., 1963), and the glucan elaborated by an imperfect fungus whose structure was reported by Johnson et al. (1963). Glucans of similar linkage have been extracted from miscellaneous plant sources (Clark and Stone, 1963). This report is the first published on production of this type of polymer by controlled fermentation. Physical properties and structure of these polymers are discussed in the following paper by Wallen, Rhodes, and Shulke (1965).

MATERIALS AND METHODS

Cultures were grown in liquid medium in indented conical flasks on a rotary shaker with an eccentricity of 5.1 cm and speed of 200 rev/min. The inoculum medium contained (per liter): 13 g of Staley's special 4S soy protein nutrient, 12 g of commercial glucose hydrate, 2 g of potassium acid phosphate, 0.5 g of magnesium sulfate heptahydrate, and 3 mg of thiamine in distilled water. The following trace elements were added (milligrams per liter, final concentration): ammonium molybdate, 0.025; magnesium sulfate hydrate, 0.03; zinc sulfate heptahydrate, 0.03; copper sulfate pentahydrate, 0.05; and ferrous sulfate heptahydrate, 2.5. Sufficient sulfuric acid was added to adjust the medium to *p*H 5.6. The soy product was kept suspended while 50-ml portions of the medium were dispensed into 300-ml conical flasks; these were sterilized at 121 C for 18 min. A piece of mycelium cut from an agar slant (medium

plus 2% agar) was incubated in shaken flasks until good growth was achieved. This growth was transferred in liquid medium to serve as inocula.

Experimental fermentations were carried out as described for inocula preparation with 100 ml of medium in 500-ml conical flasks (cotton stoppers) or 500 ml of medium in 2,800-ml Fernbach flasks (closed with woven milk filters). Unless otherwise noted, all medium components were sterilized together at 121 C for 15 or 20 min for the two flask sizes. Both rotary and reciprocal shakers were used under conditions giving the same oxygen-absorption rate (Corman et al., 1957).

Individual flasks were removed at intervals, the *p*H was recorded, and the presence or absence of glucose was determined with glucose oxidase test paper. Polymer production was gravimetrically determined by the following procedure. An equal volume of distilled water was added to the viscous liquor and, after partial disruption with a high-speed blender for 1 min, the mycelium was separated by centrifugation at 18,000 × *g* for 15 min at room temperature. After decanting the supernatant, the bulky mycelium (with an indeterminate small amount of soy solids) was transferred to a tared paper baking cup and weighed after drying overnight at room temperature and for 6 hr at 70 C. Residual glucose in the supernatant was determined by Somogyi's (1952) micro method. A 20-ml sample of supernatant was shaken in a centrifuge tube with sufficient ethyl alcohol for precipitation and was centrifuged for 10 min at about 3,000 × *g* to separate the polymer. Addition of salts was not required to obtain precipitation of the polymer, which was transferred to a tared aluminum-foil dish and dried at 70 C to constant weight. Yield was calculated as grams per liter of fermentation medium, and efficiency, as percentage by weight of glucose fermented.

In larger batches, the polymer could be harvested from the alcohol solution by lifting with a glass rod. The structureless, gelatinous mass was partially dehydrated with acetone and pressed into a flat pad, which had a fibrous structure when teased. The air-dried polymeric material was granulated through the 20-mesh screen of an intermediate-size Wiley mill. For purification, a 1 to 2% aqueous suspension of the polymer was agitated for 30 sec in a blender, dialyzed overnight against distilled water, and lyophilized.

These laboratory-scale fermentations were designed to find the optimal medium composition and conditions for pilot-scale trials.

RESULTS AND DISCUSSION

Plectania occidentalis (*NRRL 3137*). The rate of polymer formation was not greatly influenced by concentration of standard medium components, a broad range of initial *p*H, or limited changes in aeration. Apparently, biosynthesis was restricted to the formation of about 1.5% polymer, the consequent viscosity of which then limited aeration. High initial concentrations of precursor sugars were not fully utilized after the limiting viscosity was reached. Under the best conditions, 10 days or more were required for the fermentation.

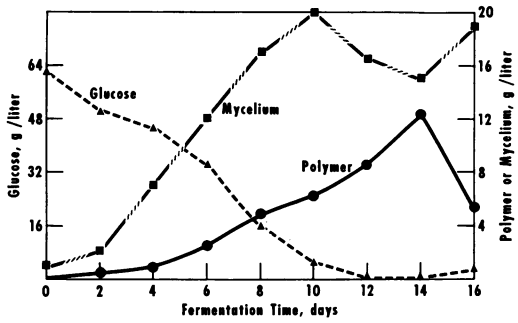

FIG. 1. *Glucan production by Plectania occidentalis NRRL 3137.*

The course of a typical fermentation is shown in Fig. 1. The 6% glucose initially supplied was completely utilized with about 20% conversion to glucan. Considerable polymer was formed after the glucose in the medium was exhausted. The weight of mycelium decreased during this period, and it may be deduced that intracellular reserve carbohydrate was converted to exocellular glucan. Polymer degradation by the organism started as soon as biosynthetic activity was complete, so that the time selected for harvest critically affected the amount of product recovered.

Glucose and xylose were equally effective as precursors of the glucan. Maltose, sucrose, and starch were poorly utilized for growth, and little polymer was formed. Although 6% glucose was sometimes completely consumed, lower concentrations often gave as much polymer. Two parts of 95% ethyl alcohol were required to precipitate the polymer, which dried to a light tan.

Staley 4S and Amber HSP 75, hydrolyzed soy proteins, were about equally effective as nitrogen sources. Corn steep liquor and wheat gluten were slightly less effective. Defatted soy flakes gave low yields. Cutter's (1946) synthetic medium, which has asparagine as the sole nitrogen source, permitted growth but no polymer formation. Staley's 4S nutrient (0.5%) was used in media in which other variables were studied. A lower concentration usually gave higher efficiencies of sugar conversion, but such fermentations were significantly slower. When a slurry of this nitrogen source was filter-sterilized, mycelial growth and sugar utilization were restricted so that only about one-half of an initial 4% glucose concentration was utilized in 10 days; however, the efficiency of polymer forma-

TABLE 1. *Carbohydrate utilization by Helotium sp. NRRL 3129*

Carbohydrate*	Mycelium	Carbohydrate used†	Polymer synthesized	Efficiency of sugar conversion
	g/liter	*g/liter*	*g/liter*	%
Arabinose	16.0	24.2	0.7	0.3
Xylose	14.0	33.0	5.6	17
Glucose	19.6	41.7	21.5	51
Fructose	12.4	41.5	18.6	45
Mannose	16.7	—	18.3	—
Galactose	12.1	26.2	1.7	7
Maltose	15.4	23.8	Trace	0
Sucrose	14.5	44.7	20.0	45
Lactose	17.6	23.8	Trace	0
Trehalose	16.8	—	Trace	0
Raffinose	13.9	24.3	1.6	6.5
Soluble starch	14.2	10.6	2.1	20

* Initial concentration, 40 g/liter.

† By reducing power as glucose equivalent; oligosaccharides acid-hydrolyzed before analysis.

tion was 45% as compared with 25 to 30% normally achieved with faster growth and sugar consumption. Optimal yields were obtained when media were initially adjusted to *p*H 6. Somewhat lower yields resulted by adjusting to *p*H 5 or 7.

The reasons for the variability observed in successive fermentations have not been completely elucidated, but differences in culture handling contributed to such variations. Routinely, 5% inoculum was used, although smaller amounts (down to 1%) gave a better conversion to product at the expense of an extended fermentation period. Variation in age of the inoculum from 2 to 7 days had little effect on the course of the fermentation. The greatest decrease in yields appeared to correlate with an increased number of transfers in liquid medium; however, the use of duplicate cultures transferred identically did not always give the same results. A temperature of 32 C resulted in poor growth and polymer production. Much better growth occurred at 28 C, but efficiency of conversion to polymer was low. Temperatures of 22 and 25 C gave less mycelium but the best yields of polymer. Since fermentations at the lowest temperature were somewhat extended, 25 C was used in most experiments.

Helotium sp. (*NRRL 3129*). Inoculum cultures were generally grown aerobically in the medium described, after transfer of mycelium from agar slants. This organism produces conidia which were also used as inocula by direct suspension in water. Alternatively, a mycelial inoculum was prepared by growing conidia in shaken liquid medium. Direct inoculation with a spore suspension delayed the course of the polymer fermentation, but a limited number of trials indicated that mycelium derived from spores gave as satisfactory an inoculum as that grown by mycelial transfer from slants.

High levels of inocula gave more growth but lower yields of polymer. A 2% inoculum gave the most rapid rate of polymer production and the best efficiency in terms of conversion of precursor to glucan. Four subcultures from the same refrigerated slant were started at different times and transferred in liquid medium thrice weekly until used as inocula. Comparative results indicated that cultures lost synthetic capacity after repeated transfers in the inoculum medium.

Twenty-two hydrolyzed protein materials were tested for their effect on polymer formation in another medium, formulated with phosphate, magnesium sulfate, thiamine, and tap water. A 20% glucose solution was heat-sterilized separately and added aseptically to give a 4% final concentration. Of nine soy protein products tested, Staley's 4S nutrient, commercial defatted soy flakes, Amber HSP 75, and Sheffield soy peptone were suitable nitrogen sources. Bourbon stillage (but not distillers' dried solubles), wheat gluten, Red Star yeast autolysate, and Amber enzyme-digested cotton seed protein were also effective nitrogen sources. Ammonium sulfate and urea, as sole nitrogen sources, gave little growth and essentially no polymer formation. The substitution of 0.2% urea for part of the hydrolyzed soy protein depressed polymer formation. In most fermentations, 0.5% soy flakes served as the nitrogen source. Whereas a black product resulted from the use of cottonseed protein, those products recovered from other media were various shades of gray or tan, with that from wheat gluten being the lightest in color. The typical gray of the crude polymer could not be removed either by dialysis or by treatment with activated carbons, but colorless material could be precipitated with alcohol after ultrasonic treatment.

Fermentations conducted at different initial acidities from *p*H 4 through 7.5 had a broad optimum for maximal growth but a narrower range near *p*H 6 for maximal sugar utilization and polymer production.

The utilization of a number of sugars was tested at a 4% concentration in a soy flakes medium. All sugars supported good growth at 72 hr, and at least 50% of each was utilized (Table 1). Appreciable glucan, however, was formed only from media containing glucose,

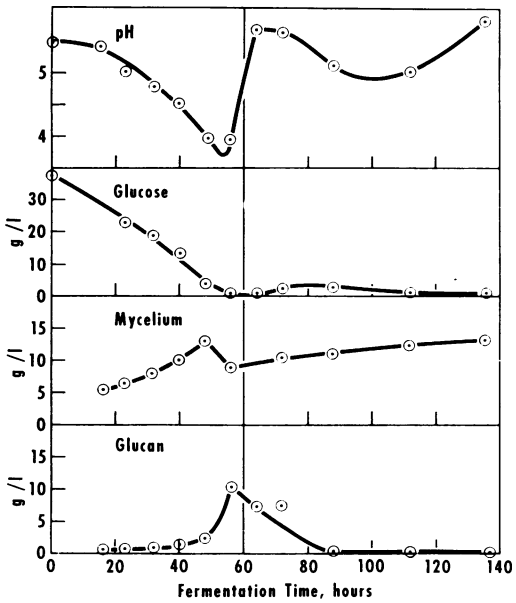

FIG. 2. *Glucan production by Helotium sp. NRRL 3129.*

fructose, mannose, or sucrose. The lack of pronounced amylase and maltase activity eliminated starch and malt syrup as substrates. In fermentations in which 3 and 4% glucose were completely utilized, efficiencies of glucose to glucan conversion in the 40 to 50% range were common; the best efficiency obtained was 65% in 64 hr.

The *Helotium* sp. in soy flake-glucose medium, agitated on rotary shakers at 22, 25, 28, and 32 C, grew very poorly at the highest temperature and only slightly better at 28 C. Good sugar utilization resulted at 22 or 25 C with conversion to polymer more rapid at 25 C, the temperature adopted for all other work. Soy flake-glucose medium prepared with tap water required no additional trace minerals or magnesium; addition of vitamins was not beneficial.

The high viscosity resulting from biosynthesis of polymer undoubtedly decreased mechanical dispersal of oxygen to the cells. A rotary shaker was more effective than a reciprocal one after appreciable concentrations of polymer had formed. Media containing the polymer frequently had a stable foam, which was not readily broken with polyglycol antifoam.

Figure 2 shows the course of a typical fermentation. Highest biosynthetic activity was achieved after the glucose had been nearly exhausted. At the same time, accumulated acid was utilized and mycelium weight decreased abruptly. As with *P. occidentalis*, it is believed that these events can be best explained by assuming the conversion of cellular carbohydrate reserves to exocellular polymer. When cell reserves were exhausted, the polymer was hydrolyzed. Presumably, the organism secretes an exocellular glucanase under these conditions. Occasionally, free glucose occurred in the medium at this time and was used by the organism either for renewed growth or to rebuild cellular reserves.

The harvest time is critical; a significant proportion of the total polymer is formed after the supplied sugar is exhausted or nearly so. If harvest is delayed too long, depolymerization causes severe reductions in the amount of recoverable polymer. Extrapolation of analytical data for residual glucose in the medium appears to be the best way to anticipate optimal harvest time. A sharp rise in *p*H signals the completion of glucan formation.

Approximately 95% of the total polymer was recovered by the method described for the determination of polymer yield. The addition of 0.5 volume of ethyl alcohol to the diluted, centrifuged medium gave rapid and complete precipitation; 0.4 volume gave a voluminous precipitate only after standing for several hours. Disruption of the mycelial aggregates with a blender greatly facilitated separation of polymeric material. Conversely, about one-half of the total polymer adhered to mycelium separated by centrifugation of diluted culture liquors; such adherent polymer could be recovered by subsequent washing of the mycelium with water. Heating to 70 C and centrifuging at that temperature showed promise as a simplified recovery procedure for large-scale use; in addition, this heat treatment can be assumed to inactivate depolymerization enzyme activity.

ACKNOWLEDGMENTS

We are grateful to Dorothy Coffman for excellent technical assistance, to R. G. Benedict for the collection and isolation of the organisms used, and to J. J. Ellis and C. R. Benjamin for assistance in identification of the organisms.

LITERATURE CITED

CLARK, A. E., AND B. A. STONE. 1963. Chemistry and biochemistry of β-1,3 glucans. Rev. Pure Appl. Chem. **13**:139–156.

CORMAN, J., H. M. TSUCHIYA, H. J. KOEPSELL, R. G. BENEDICT, S. E. KELLEY, V. H. FEGER, R. G. DWORSCHACK, AND R. W. JACKSON. 1957. Oxygen absorption rates in laboratory and pilot-plant equipment. Appl. Microbiol. **5**:313–318.

CUTTER, V. M. 1946. The genus Cunninghamella (Mucorales). Farlowia **2**:321–343.

JOHNSON, J., JR., S. KIRKWOOD, A. MISAKI, T. E. NELSON, J. V. SCALETTI, AND F. SMITH. 1963. Structure of a new glucan. Chem. Ind. (London), p. 820–822.

NELSON, T. E., J. V. SCALETTI, F. SMITH, AND S. KIRKWOOD. 1963. The use of enzymes in structural studies of polysaccharides. I. The mode of attack of a β-D-(1 → 3)-glucanase on laminarin. Can. J. Chem. **41**:1671–1678.

REESE, E. J., AND M. MANDELS. 1959. β-D-1,3 Glucanases in fungi. Can. J. Microbiol. **5**:173–185.

SOMOGYI, M. 1952. Notes on sugar determination. J. Biol. Chem. **195**:19–23.

WALLEN, L. L., R. A. RHODES, AND H. R. SHULKE. 1965. Physical properties and chemical composition of β-glucans from fleshy fungi. Appl. Microbiol. **13**:272–278.