

1987

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Recommended Citation

Dunleavy, J. M. and Gobelman-Werner, K. S. (1987) "Method of Extracting Starch from Bacteria," *Proceedings of the Iowa Academy of Science*, 94(4), 107-110.
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Method of Extracting Starch from Bacteria¹

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Corynebacterium kutscheri required 10 days of growth on semisolid medium to accumulate intracellular starch, but when the same medium was used as a broth, only 1 day of growth was required. *C. kutscheri* synthesized starch when amylose was added to nutrient agar in the substrate, but did not do so when amylopectin was substituted for amylose. Dimethyl sulfoxide (DMSO) was superior to water as a cell wash for removing substrate starch from cells before chemical treatment to remove intracellular starch. Bacterial starch was extracted from *C. kutscheri* cells by destroying cell walls with lysozyme and sodium lauryl sulfate, removing cellular debris by centrifugation, and precipitating the starch from the supernatant with butanol. A qualitative method for the separation of this starch into amylose and amylopectin fractions is described. Use of DMSO in cell washes established that the bacterial starch molecule was too large to pass through the membrane of the bacterial cell because DMSO passes through the cell membrane and cells gave a positive test for starch after the treatment.

INDEX DESCRIPTORS: bacteria, starch, amylose, amylopectin, *Corynebacterium kutscheri*.

The polysaccharide glycogen is a primary energy storage product in liver and muscle of animals, whereas its counterpart in plants is starch. Starchy foods have always been an item in the diet of man, and use of starch for sizing fibers from the papyrus plant into a writing material began about 3000 BC. Starch is composed of glucose units joined in α -1, 4-linkages, and the repeating disaccharide unit is maltose. Plant starches are a mixture of two types of compounds that are separable. Amylose is a long, unbranched chain, whereas amylopectin is a branched-chain polysaccharide with one terminal glucose occurring for every 24 to 30 glucose residues (14).

Bacterial synthesis of polysaccharides is common, but bacterial synthesis of starch is relatively rare. Production of starch from glucose-1-phosphate has been observed in a mutant strain of *Escherichia coli* (5), *Corynebacterium diphtheriae* (1), *Neisseria* (7), certain *Streptococcus* strains (1), and *Clostridium* isolated from pig cecum (11). Glucose could not be substituted for glucose-1-phosphate in the substrate for starch synthesis with any of these organisms.

Maltose, maltotriose, dextrans, glycogen, amylose, amylopectin, and starches were suitable substrates for synthesis of starch-like material by rumen streptococci (3, 4, 9). The process whereby substrate starch is converted to intracellular starch has been the objective of several studies. It is generally conceded that the molecule is too large to pass through the bacterial membrane (2). Monod and Torriani (10) believed that the synthesis of intracellular starch-like material was mediated by an enzyme, amyloamylase. In subsequent years, amylosucrase (8), transglycosylase (6), and amyloamylase (2) were suggested names for the enzyme.

Such terms as "iodophilic material" and "starch-like material" were used in the literature because researchers were uncertain that the intracellular product was starch. Two primary difficulties were involved in establishing that the intracellular product was starch: 1) The starch molecule was believed to be too large to pass through the bacterial membrane, and 2) starch is a relatively fragile molecule and withstands chemical treatment poorly. Sonication has been used to free starch from cells (2), but this technique is useful for only relatively small quantities of bacterial cells. Bacterial production of starch may have potential in biotechnology, and techniques applicable to large-scale harvesting of such starch might be useful. The objective of this

study was to devise a chemical method of extracting starch from bacterial cells.

MATERIALS AND METHODS

Materials

Amylopectin (potato), amylose (potato), dimethylsulfoxide (DMSO), lysozyme (3X crystalline), potato starch, and sodium lauryl sulfate were purchased from Sigma Chemical Co., St. Louis, Mo.²

Microorganism, Media, and Growth

Corynebacterium kutscheri (Migula) Bergey *et al.* (ATCC 15677) was obtained from the American Type Culture Collection, Rockville, Md. Nutrient agar with 0.3% potato starch added (NAPS) was used in petri dishes for maintenance and for observation of starch formation by *C. kutscheri*. Plates were incubated for 10 days at 26C and tested for starch formation by flooding the colonies with Gram's iodine solution. Nutrient broth (BBL) with 0.3% potato starch added (NBPS) was used for bacterial starch production. The medium was dispensed in 250 ml quantities in 500-ml flasks, which were placed on a gyratory shaker after sterilization and inoculated with *C. kutscheri*, which was grown at 30C for 24 hr before use. Seven flasks (1.75 liters of NAPS) yielded 5 to 6 g of wet packed cells.

For the study of utilization of amylose and amylopectin on starch production by *C. kutscheri*, two media were used. In the first, amylose (0.3%) was dissolved in cold 0.1 N sodium hydroxide and neutralized with 1 N hydrochloric acid immediately before it was added to nutrient agar; in the second, amylopectin (0.3%) was added to nutrient agar. All sterilization was performed in an autoclave at 121C for 15 min.

Spectrophotometric Detection of Starch

In an experiment to test the sensitivity of detecting presence of starch spectrophotometrically, concentrations of 0.3% and 1% of potato starch in 10 ml of water contained in test tubes were diluted 10-fold in each of seven steps. Three drops of Gram's iodine per 10 ml of solution was added to each dilution of each concentration of starch before the absorbance (620 nm) of each dilution was read in a spectrophotometer. Another set of dilutions was treated similarly, except that no iodine was added to the tubes.

Cell Washes

The effectiveness of washing cells of *C. kutscheri* grown in NBPS medium for 24 hr at 30C, with successive water washes, was tested by growing the organism in duplicate flasks as described for bacterial starch production. Cells from 250 ml of medium harvested in bottles by centrifuging for 30 min at 6,000 \times g, were combined and transfer-

¹Joint contribution: Agricultural Research Service, U.S. Department of Agriculture, and Journal Paper No. J-12445 of the Iowa Agriculture and Home Economics Experiment Station, Ames, IA 50011. Project 2475.

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red to a 40-ml centrifuge tube. Cells were washed by resuspending in 20 ml sterile distilled water with a mechanical agitator, and centrifuging for 30 min at $7,800 \times g$. The supernatant liquid was retained, and this cycle was repeated six times. The control consisted of uninoculated medium which was treated in the same way as the *C. kutscheri*-inoculated medium. Absorbance of the supernatant liquids in the spectrophotometer was obtained at 620 nm after addition of three drops of Gram's iodine per 10 ml of liquid.

The effectiveness of a dimethyl sulfoxide (DMSO) wash in removing starch particles from the bacterial pellet was determined by substituting 20 ml of DMSO for water in the second wash of the procedure just described, except that only three additional water washes followed the DMSO wash. Pelleted cells of *C. kutscheri*, which had been water-washed, were examined microscopically in Gram's iodine to determine presence of potato starch from the medium. The same procedure was used to determine the effect of the DMSO-wash on presence of bacterial-produced starch in cells of *C. kutscheri*. Starch is readily soluble in DMSO, but forms a colloidal solution in water (13).

Extraction of Starch from Bacteria

Cells were prepared for bacterial starch extraction by using the wash procedure described for the DMSO-wash experiment. Cellular destruction was achieved by treating 5 g of wet, pelleted cells with 20 mg of lysozyme in 9 ml of water at 37C for 24 hr, adding 2 ml of a 25% sodium lauryl sulfate solution, and heating to 60C for 10 min. The mixture was cooled to room temperature, 150 ml of DMSO were added, and the mixture was stirred overnight. (Dry starches must be wet with water before they are soluble in DMSO). Cellular debris was removed by centrifugation for 30 min at $400 \times g$. Butanol (300 ml) was added to the supernatant liquid, and the starch precipitate was allowed to settle overnight, or longer, if necessary. The starch was pelleted by centrifugation for 30 min at $7,800 \times g$. If bacterial starch is the final product desired, the procedure can be stopped at this point.

The starch was further purified by heating to 100C in 200 ml of water in an atmosphere of nitrogen, cooling to 60C, adding 0.1 g thymol, and allowing to stand for 3 days to precipitate amylose. The amylose was pelleted by centrifuging for 1.5 hours at $15,000 \times g$. The supernatant liquid contained the amylopectin portion of the starch. Thymol was removed from the supernatant by transferring the liquid to a separatory funnel, adding an equal volume of absolute ethyl alcohol, 6 ml of ethyl ether, and shaking before removing the lower liquid from the funnel. We allowed 3 to 4 days for the amylopectin to precipitate and then pelleted the precipitate by centrifuging for 15 min at $7,800 \times g$.

Control starch was composed of a mixture of potato amylose (200 mg) and potato amylopectin (200 mg) and was subjected to the same treatments as bacterial cells, beginning with the lysozyme treatment.

RESULTS AND DISCUSSION

C. kutscheri required 10 days growth on semisolid NAPS medium before colonies stained uniformly blue when flooded with Gram's iodine; however, when grown in the same medium as a broth, centrifuged, washed cells stained blue after 24 hr of growth. Such cells, when examined with a microscope, had one to several blue granules per cell. Bacterial growth in a liquid medium has the advantages of rapid starch synthesis by bacterial cells as well as ease of harvesting cells for starch extraction.

Because the growth medium contained starch, it was essential to remove all medium starch from the cells before beginning starch extraction experiments. We tested the sensitivity of detecting starch spectrophotometrically. Absorbance (620 nm) of the starch-iodine

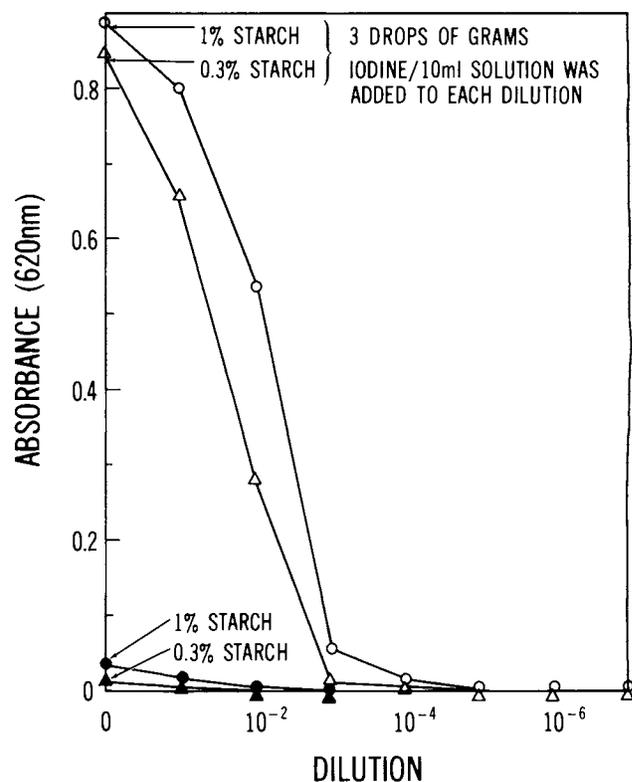


Fig. 1. Reduction in the absorbance of the starch-iodine complex of two concentrations (0.3% and 1%) of starch in water as a result of a series of 10-fold dilutions with water contrasted to that occurring in dilutions of the same starch concentrations, but without iodine.

complex in both 0.3% and 1% potato starch solutions fell sharply from the original concentrations through the 10^{-3} dilution, at which point both curves leveled (Fig. 1). Starch concentration of the 1% solution at this dilution was $10 \mu\text{g}$ per ml, and that in the 0.3% solution was $3 \mu\text{g}$ per ml. This procedure provided good sensitivity for detecting low concentrations of starch and was used to monitor wash water in subsequent experiments.

The attempt to remove medium potato starch from the exterior of the cells with water washes was unsuccessful. Although absorbance of the starch-iodine complex in wash water was greatly reduced after seven washes with water (Fig. 2), we observed microscopically that starch particles were trapped among cells in the pellet after the seventh water wash. A small starch pellet was formed in the control (NBPS medium alone) after the first and second centrifugations, but no pellet was observed after subsequent centrifugations.

An improved cell wash procedure utilized DMSO, that completely dissolved colloidal starch. Starch was present in control wash water before, but not after the DMSO wash (Table 1). After the DMSO wash, three cell washes were used to remove DMSO from the cells. Microscopic examination of the cells after the third water wash revealed no extra-cellular starch particles, and when Gram's iodine was applied to the cells, they stained blue, indicating that DMSO did not remove intracellular starch. This observation also established that the bacterial starch molecule was too large to pass through the membrane of the bacterial cell because DMSO passes through the cell membrane (12, 13).

The extraction procedure was tested in three separate assays. *C. kutscheri* formed intracellular starch composed of both amylose and amylopectin (Table 2) when cells were grown in NBPS medium and washed with DMSO. The mean yield of amylopectin was 53% greater than that of amylose. Mean yields were 4.3 ± 0.4 and $6.6 \pm 2.9 \text{ mg/g}$

Table 1. Absorbance of cell wash water from a 24-hr culture of *C. kutscheri* grown in NBPS medium and that in wash water from a control of centrifuged NBPS medium alone.

| Wash number | Absorbance (620 nm) | |
|-------------|--|---|
| | Cell wash water from <i>C. kutscheri</i> grown in NBPS ^a medium | Wash water from centrifuged NBPS medium (control) |
| | Before DMSO ^b wash | |
| 1 | 0.71 ^c | 0.86 |
| | After DMSO wash | |
| 2 | 0.03 | 0.03 |
| 3 | 0.02 | 0.02 |
| 4 | 0.02 | 0.02 |

^aNutrient broth plus 0.3% potato starch

^bDimethyl sulfoxide

^cData are means of two replications

of cells for amylose and amylopectin, respectively. The mean percentage recovery of amylose and amylopectin potato starch controls was 80 ± 16 and $79 \pm 18\%$, respectively. During starch processing, the potato starch control precipitated in 1 day, but the bacterial starch formed a finer precipitate and required 3 days. This quality of the bacterial starch may have contributed to a firmer pellet and lower standard deviation values for the bacterial starch as contrasted to those for potato starch. This method of starch extraction is not quantitative because, after centrifugation, the starch pellets varied considerably in compactness. Pellets tended to be loosely packed near the starch-liquid interface, which sometimes led to loss of starch when the supernatant was decanted. Although all the control starch (50% amylose and 50% amylopectin) in assay number 1 was reclaimed, there was no assurance that some bacterial starch in the same assay was not lost.

Bacterial starch displayed the same properties as the control potato starch. It formed a colloidal solution in boiling water, was soluble in DMSO, was precipitated from solution by butanol, and formed a starch-iodine complex that gave an absorbance maxima at 620 nm. The amylose fraction of bacterial starch gave a dark blue color (characteristic of amylose) with Gram's iodine, whereas the amylopectin fraction gave a magenta color (characteristic of amylopectin), which has been observed by others (14). Additional evidence of the identity of the final product was obtained by treatment of *C. kutscheri*-produced amylose and potato amylose with both heated and unheated

Table 2. Mean yield of amylose and amylopectin from *C. kutscheri* and percentage of amylose and amylopectin recovered from control starch in three separate assays.

| Assay number | Bacterial starch | | Control starch ^a | |
|--------------|-----------------------|---------------------------|-----------------------------|----------------------------|
| | Amylose mg/g cells | Amylopectin mg/g cells | Amylose % recovered | Amylopectin % recovered |
| 1 | 4.2 ^b | 9.8 | 100 | 100 |
| 2 | 4.0 | 4.0 | 61 | 57 |
| 3 | 4.8 | 6.1 | 80 | 79 |
| Mean | 4.3 ± 0.4^c | 6.6 ± 2.9 | 80 ± 16 | 79 ± 18 |

^aComposed of a mixture of 200 mg each of amylose and amylopectin

^bData are means of two replications

^cStandard deviation

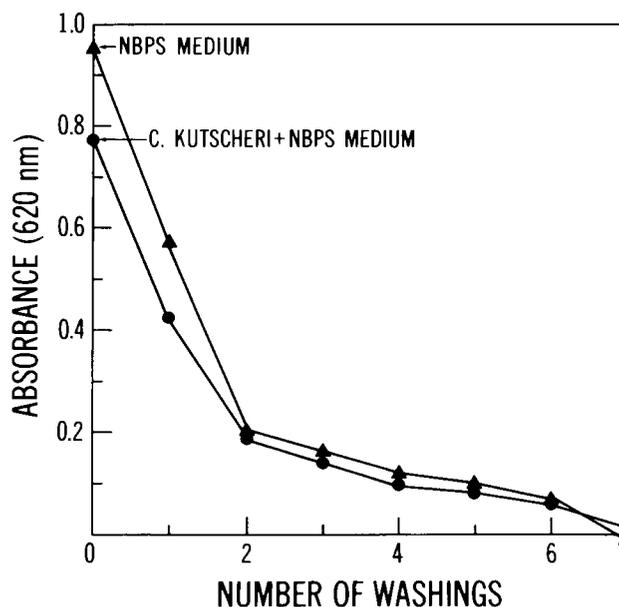


Fig. 2. Reduction in the absorbance of the starch-iodine complex in 24-hr-cultures of *C. kutscheri* in nutrient broth-potato starch medium (NBPS) resulting from a series of centrifugations and water washes compared with that occurring in medium only. One drop of Gram's iodine per 10 ml of solution was added to supernatant broth after centrifugation (0 washing) and to supernatant wash water after centrifugation in each subsequent wash.

amylase. This treatment resulted in destruction of both bacterial amylose and potato amylose by the unheated enzyme, but both types of amylose were unaffected by the heated enzyme (Fig. 3).

When *C. kutscheri* was grown on nutrient agar alone or with amylopectin added, no starch accumulated in cells. When amylose was added to nutrient agar, starch was accumulated, as indicated by appearance of a dark blue color when cells were treated with Gram's iodine (Table 3). Additionally, when cells were cultured on nutrient agar containing potato starch (both amylose and amylopectin), cells stained blue with Gram's iodine. Thus, amylose in the medium supported starch synthesis by *C. kutscheri*, but amylopectin did not.

Carrier and McClesky (2) reported that six species of *Corynebacterium*, including *C. kutscheri*, accumulated amylose when the substrate included either potato amylose or potato amylopectin. They observed that Ramilin (a commercial amylopectin from potato) contained about 5% amylose and that the contaminating amylose might explain the presence of amylose in bacteria grown with this product in the medium. Our observation that *C. kutscheri* could not utilize amylopectin to form either amylose or amylopectin confirms this observation, at least for *C. kutscheri*.

Our observation that *C. kutscheri* synthesizes both amylose and amylopectin differs from the report of Carrier and McClesky (2) that this bacterium forms only amylose. These researchers grew the bacterium on a semisolid medium, whereas we used a broth. Except for their addition of agar, the two media used for starch production were the same. They reported that a pH between 6.0 and 8.0 and temperatures between 20 and 37°C had little effect on bacterial starch synthesis. That there is a large difference between the two types of growth is illustrated by the fact that growth of *C. kutscheri* in broth produced bacterial starch nearly 10 times faster than growth on the same semisolid medium. Whether this bacterium synthesized both amylose and amylopectin from reconstituted amylose alone in broth culture or utilized both amylose and amylopectin remains to be



Fig. 3. Production of dark blue starch-iodine complex resulting from treating colloidal solutions of potato amylose (top row) and *C. kutscheri*-produced amylose (bottom row) with (left to right) no treatment, Gram's iodine, amylose plus Gram's iodine, and amylose heated to 100C plus Gram's iodine.

determined. However, the availability of our method of extraction of bacterial starch should speed the clarification of some of these perplexing problems.

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Table 3. Effect of the type of starch in the medium on the color of the medium and bacterial colonies of *C. kutscheri*.

| Medium | Color change after Gram's iodine | |
|--------------------|----------------------------------|---------------------|
| | Medium | Colony ^a |
| Nutrient agar (NA) | none ^b | none |
| NA + potato starch | dark blue | blue |
| NA + amylopectin | magenta | none |
| NA + amylose | dark blue | dark blue |

^aAfter 10 days of bacterial growth

^bAll observations were based on five replications

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