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TECHNICAL MANUSCRIPT 6

EFFECTS OF SUGARS ON THE
SURVIVAL OF SERRATIA MARCESCENS
AFTER FREEZE DRYING OR
AEROSOLIZATION AT
UNFAVORABLE HUMIDITY

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ABSTRACT

Suspensions of Serratia marcescens were subjected to freeze drying or to aerosolization at unfavorable humidity levels. The survival of the cells during these treatments was markedly improved in the presence of common sugars, but no one sugar stabilized the cells against both stresses. The protective effects of the sugars were correlated with their penetrability into cells: minimally penetrable (MP) sugars stabilized cells against aerosolization and freely penetrable (FP) sugars stabilized cells during freeze drying. These results were attributed to the modifications of intracellular water content induced by the presence of the sugars in the cell suspensions.

CONTENTS

| | |
|--|----|
| Abstract | 3 |
| I. INTRODUCTION | 7 |
| II. MATERIALS AND METHODS | 7 |
| A. Cell Suspensions | 7 |
| B. Freeze Drying | 8 |
| C. Oxygen Uptake Studies | 8 |
| D. Aerosol Testing | 9 |
| E. Penetrability | 9 |
| F. Sugar Determinations | 11 |
| III. RESULTS | 11 |
| IV. DISCUSSION AND CONCLUSIONS | 14 |
| Literature Cited | 17 |

TABLES

| | |
|--|----|
| I. Survival of <u>S. marcescens</u> Cells after Freeze Drying or Aerosolization at 30% RH in the Presence of 1% of Individual Sugars | 12 |
| II. Effects of Mixtures of Raffinose and Sorbose on the Survival of <u>S. marcescens</u> Cells Aerosolized at 30% RH | 13 |
| III. Survival of <u>S. marcescens</u> Cells Freeze Dried in the Presence of Mixtures of Sorbose and Raffinose | 13 |

I. INTRODUCTION

When suspensions containing vegetative bacteria were aerosolized at high relative humidity (RH), most of the viable cells remained alive. When such suspensions were either aerosolized at low RH, or freeze dried, most of the cells were killed. Much of the water in cell suspensions was removed by evaporation during freeze drying or aerosolization at low RH; this loss of water may have killed the cells.

In an effort to counteract this dehydration process, various solutes were added to cell suspensions before treatment. It was found that adding sugars to suspensions of Serratia marcescens markedly enhanced the ability of the cells to survive freeze drying or aerosolization at low RH. The results of this investigation are reported here.

II. MATERIALS AND METHODS

A. CELL SUSPENSIONS

The S. marcescens cells used in these studies were grown in aerated culture at 30°C on a tryptose-glucose-phosphate medium. After the culture reached its maximum population level, its cells were centrifuged and re-suspended in a small volume of culture supernate. The resulting slurry contained about 10×10^{11} viable cells per milliliter; it was frozen at -50°C into small pellets and stored at -78°C until used.

Suspensions were prepared for freeze drying or aerosolization by mixing one volume of thawed pellets with 49 volumes of solution, yielding suspensions containing 20×10^9 viable cells per milliliter. The dilution process was often performed in two stages: (a) the thawed cell concentrate was mixed with 24 times its volume of water and shaken aerobically at 25°C for three hours; (b) portions of the shaken suspensions were then diluted with equal volumes of the sugar solutions to give the desired sugar and cell concentrations.

The suspensions prepared for aerosol testing were incubated at 25°C for one or two hours after addition of the sugars, then placed at 4°C. Aerosol tests were made on samples of these suspensions during each of the following three days.

Suspensions prepared for aerosol testing were often used for freeze-drying tests. Suspensions to which oxidizable sugars* were added, however,

* As defined subsequently, those sugars that accelerated the oxygen uptake rate of cell suspensions.

were always freeze dried between one and two hours after the combination of cells with solutes. Variations in the technique of preparing cell suspensions had no detectable influence on the survival of cells subjected to freeze drying.

B. FREEZE DRYING

Suspensions were freeze dried in a batch-type Waffle Iron dryer built at this installation. This unit had the following special features: (a) fluids could be circulated through passages in the bedplate to control its temperature, (b) the bedplate was counterbored to accept 24 vaccine vials, and (c) at the end of a run, the vials containing the freeze-dried material could be stoppered while still under vacuum.

At the start of a run, coolant was circulated through the bedplate to bring it to -50°C . Duplicate or triplicate one-milliliter samples of cell suspensions, each in a 15-milliliter vial, were then placed on the bedplate and frozen. After ten minutes, the chamber of the dryer was sealed, evacuated, and brought to a pressure of approximately 40 microns of Hg (± 10 microns). The temperature of the plate holding the vials was raised to 40°C as soon as possible after the operating pressure was reached (within 15 minutes), and the 40°C temperature was maintained for the remainder of the three-hour drying cycle. At the end of this time, the bottles were stoppered under vacuum, removed from the drying chamber, and kept at 4°C . While still under vacuum, the samples were reconstituted with distilled water (within two hours after removal from the dryer). The number of cells surviving freeze drying and reconstitution was estimated from the colony counts obtained with a standard surface-plating technique, using distilled water dilution blanks and Difco Casitone agar plates.

C. OXYGEN UPTAKE STUDIES

The ability of S. marcescens cells to oxidize the sugars added to suspensions prepared for testing was evaluated in a Warburg respirometer by the techniques of Umbreit, Burris, and Stauffer.^{1/*} The effect of the solute on cellular respiration was determined in the absence of buffer or other extraneous solutes. Under these conditions, some sugars (defined as "oxidizable") caused an immediate and marked increase in the rate of oxygen uptake of cells in water suspension. Others, including some known to support growth as the sole source of carbon in a chemically defined medium (lactose and sucrose), had no effect on this rate. The pH of cell suspensions containing sugars was also determined after 72 or 96 hours of storage at 4°C , in order to detect the possible delayed decomposition of the sugar by the cells after a protracted period of contact.

* See Literature Cited.

D. AEROSOL TESTING

Aerosol tests were performed in two 1280-liter Plexiglas chambers, similar to those described by Ray.^{2/} Each chamber was equipped with (a) an ultrasonic two-fluid nozzle (Hartmann whistle) for aerosol generation, (b) a mixing fan, (c) sampling ports, (d) a wet-and-dry-bulb thermometer unit for determining relative humidity (RH), and (e) an exhaust blower. Each capsule was also equipped with accessory units for generating humidified or dehumidified air as required. In all these tests, five milliliters of cell suspension were disseminated into air at 24° to 27°C and 30 per cent RH, whereupon the RH was spontaneously increased to about 47 per cent. Aerosol samples were withdrawn through all-glass impingers^{3/} for one-minute time periods at two and sixteen minutes (midpoints) after aerosol generation. The observed viable cell population per milliliter of fluid in the aerosol sampler was determined from the colony counts obtained with the standard surface-plating technique.

Maximum possible recovery (100 per cent) of viable cells from aerosols was calculated by the following formulae:

$$\begin{aligned} &\text{Maximum possible recovery (viable cell numbers/ml of fluid in sampler)} && (1) \\ &= \frac{\text{number of organisms disseminated}}{\text{volume of aerosol chamber (liters)}} \cdot \frac{\text{liters of aerosol withdrawn}}{\text{volume of sampling fluid (ml)}} \end{aligned}$$

$$\% \text{ recovery} = \left(\frac{\text{observed}}{\text{maximum possible}} \right) \begin{array}{l} \text{viable cell population per ml of fluid} \\ \text{in the sampler} \end{array} \quad (2)$$

Viable cell recovery levels obtained from aerosolized cell suspensions containing various sugars sometimes varied considerably from month to month; summer-to-winter variation was the most obvious effect observed. Nevertheless, the recovery patterns obtained from these tests were so reproducible that the apparent trial-to-trial variability of the data did not obscure their interpretation.

E. PENETRABILITY

The penetrability of sugars into S. marcescens cells was determined by a technique "based on the concept that any cell volume which will act to dilute added substrate is permeable to that substrate. Therefore, the permeable fraction of the total cell volume should be measurable by the degree of this dilution when a known substrate concentration is added to a known large volume of cells."^{4/} This method was originally credited to Conway and Downey.^{5/}

In these experiments, 4.5 milliliters of packed* S. marcescens cells were resuspended in 3.0 milliliters of a one per cent sugar solution, so

* At a force of 20,000 times gravity for 15 minutes.

that these preparations contained 30,000 micrograms of added sugar. After 15 minutes of incubation at 25°C, the suspension was centrifuged and the sugar concentration in its supernate determined. Packed cell preparations resuspended in water were assayed in parallel to determine the concentration of sugar-like reactive material spontaneously released by the cells.

From these data, the penetrability of the sugar was determined by the following formulae:

$$S = \frac{30,000 \mu\text{g}}{C_X - C_B} \quad (3)$$

$$V_A = \frac{100 \cdot S - 3.0}{V_T} \quad (4)$$

$$V_T = V_I + V_X \quad (5)$$

$$P = \frac{V_A - V_X/V_T(\%)}{100(\%) - V_X/V_T(\%)} \quad (6)$$

where

C_X = measured sugar concentration ($\mu\text{g/ml}$) in supernate of packed cell preparation, to which 3.0 milliliters of one per cent sugar solution were added.

C_B = measured sugar concentration ($\mu\text{g/ml}$) in supernate of packed cell preparation, to which 3.0 milliliters of water were added.

S = apparent total space (milliliters) available for dilution of test solute.

V_A = apparent percentage of volume of packed cell preparation available for solute dilution.

V_T = total volume (milliliters) of packed cell preparations.

V_I = cellular volume (milliliters) in packed cell preparations.

V_X = extracellular volume (milliliters); space available in packed cell preparations for diluting solutes not penetrable into individual cells. Using a presumably impenetrable solute, a clinical dextran with molecular weight of 75,000, V_X was found to equal 20 per cent of V_T .

P = apparent percentage of cellular volume available for dilution of test solute, or apparent cellular penetrability of test solute.

Sugars were arbitrarily divided into two classes on the basis of the values of P , minimally penetrable (MP) sugars yielding values between 22 and 39 per cent, and freely penetrable (FP) sugars yielding values between 54 per cent and 86 per cent.

F. SUGAR DETERMINATIONS

The concentrations of sugars in the supernate of cell suspensions were determined by the anthrone- H_2SO_4 reagent.^{6/} Reproducible results were not obtained with this assay technique unless the cells were first washed in four changes of distilled water over a period of five hours. Only after this processing did the background concentration of anthrone-reactive material in untreated cell suspensions (control) fall to a low and reproducible level.

III. RESULTS

Samples of cell suspensions containing one per cent (final concentration) of various sugars were subjected to freeze drying or aerosolization, and the numbers of cells surviving these treatments were determined. Other untreated samples were analyzed to determine (a) the apparent penetrability of the sugars into the cells, (b) the oxidizability of the sugars by the cells as indicated by stimulation of the rate of oxygen uptake, and (c) the pH of the cell suspensions after storage at 4°C for 72 hours. The results of these tests are presented in Table I, which indicates the following:

(a) All of the monosaccharides stabilized the cells against freeze drying; all of the oligosaccharides stabilized cells against aerosol exposure. No sugar protected the cells against both stresses.

(b) Oxidizable sugars were identified by their stimulation of cellular oxygen uptake and by their effects upon the pH of stored suspensions. The oxidizable sugars, however, were heterogeneous with respect to both their molecular sizes and their effect on the viability of stressed cells.

(c) The penetrability of the sugars was observed to be correlated with their ability to protect the cells; MP sugars were effective only during aerosolization and FP sugars were effective only during freeze drying.

Examination of these data suggested that suspensions containing suitable amounts of FP and MP sugars might possess stability during both freeze drying and aerosolization. This possibility was investigated by experiments performed on cell suspensions containing mixtures of the nonoxidizable sugars sorbose (FP) and raffinose (MP). The viable cell recovery values obtained after aerosolization of these suspensions are shown in Table II. These data were evaluated primarily on the basis of the 16-minute survival levels.

TABLE I. SURVIVAL OF *S. MARCESCENS* CELLS AFTER FREEZE DRYING OR AEROSOLIZATION AT 30% RH IN THE PRESENCE OF 1% OF INDIVIDUAL SUGARS

| | Oxygen Uptake Rate at 30°C, $\frac{a}{\mu}$ /min | pH ^b / after 72-96 hr at 4°C | Apparent Space Available for Solute Dilution ^b , % | Per Cent Survival after Freeze Drying ^c /2 min | Per Cent Survival ^d / after Aerosolization at 30% RH 16 min |
|-------------|--|---|---|---|--|
| Water | 0.4-0.8 | 6.8-7.4 | - | 5 | 3-9 |
| d-xylose | 15.0 | 3.5 | 77 | 34 | 2 |
| d-arabinose | 0.7 | 7.1 | 63 | 35 | 3 |
| l-arabinose | 9.5 | 3.5 | 85 | 31 | 3 |
| d-glucose | 19.0 | 3.5 | 78 | 50 | 1 |
| d-mannose | 13.0 | 3.8 | 76 | 45 | 0.8 |
| d-galactose | 15.0 | 3.2 | 54 | 49 | 1 |
| d-fructose | 6.0 | 5.1 | 55 | 32 | 6 |
| l-sorbose | 0.6 | 6.8 | 59 | 34 | 14 |
| lactose | 0.6 | 4.6 | 27 | 4 | 31 |
| sucrose | 0.6 | 7.1 | 33 | 8 | 31 |
| maltose | e/ | 6.8 | 33 | 8 | 33 |
| cellobiose | 1.0 | 4.6 | 26 | 3 | 21 |
| turanose | 0.6 | 7.1 | 24 | 7 | 34 |
| trehalose | 0.6 | 7.0 | 21 | 5 | 26 |
| melibiose | 15.0 | 3.2 | 39 | 11 | 10 |
| mellezitose | 0.6 | 7.3 | 22 | 5 | 26 |
| raffinose | 0.6 | 7.3 | 23 | 5 | 34 |
| dextran | Not done | 7.1 | 0 | 3 | 21 |

a. Mean of at least two replicates.

b. Mean of at least three replicates.

c. Mean of at least three replicates, two or three samples per replicate.

d. Mean of at least three replicates, three trials per replicate.

e. Falls rapidly to 0.6.

TABLE II. EFFECTS OF MIXTURES OF RAFFINOSE AND SORBOSE ON THE SURVIVAL OF S. MARCESCENS CELLS AEROSOLIZED AT 30% RH

| SORBOSE CONCENTRATION, % | RAFFINOSE CONCENTRATION | | | | | |
|-----------------------------|---|--------|------------|--------|------------|--------|
| | 0% | | 1% | | 10% | |
| | Per Cent Survival ^{a/} of Aerosolized Cells after: | | | | | |
| | 2 min | 16 min | 2 min | 16 min | 2 min | 16 min |
| 0 | 13 | 0.6 | 42 | 12 | 39 | 16 |
| 0.1 | 11 | 1 | 39 | 10 | 38 | 16 |
| 1.0 | 21 | 0.6 | 28 | 1 | 36 | 11 |
| 5.0 | 15 | 0.05 | (Not done) | | (Not done) | |

a. Values are means of two replicates, three trials per replicate.

As observed previously, raffinose (alone) was an effective aerosol stabilizer; sorbose (alone) was not. Equivalent stability was induced by one per cent and ten per cent raffinose, alone or in the presence of 0.1 per cent sorbose. Raffinose in combination with one per cent sorbose, however, retained its stabilizing activity at ten per cent concentration, but not at one per cent.

The effects of mixtures of the same sugars on the survival of freeze-dried cells are shown in Table III. Raffinose (alone) did not stabilize the cells at any concentration. Sorbose at the 0.1 per cent level induced some stability, but its effects were abolished in the presence of one per cent or more of raffinose. One per cent sorbose induced stability that was maintained in combination with one per cent, but not five per cent or more, of raffinose. Sorbose at the five per cent level was a poor stabilizer, but the addition of one per cent or more of raffinose restored to it the activity characteristic of lower sorbose concentrations.

TABLE III. SURVIVAL OF S. MARCESCENS CELLS FREEZE DRIED IN THE PRESENCE OF MIXTURES OF SORBOSE AND RAFFINOSE

| Sorbose Concentration, % | PER CENT SURVIVAL ^{a/} | | | |
|--------------------------|---------------------------------|----|----------|-----|
| | Raffinose Concentration | | | |
| | 0% | 1% | 5% | 10% |
| 0 | 5 | 8 | 9 | 8 |
| 0.1 | 20 | 8 | Not done | 5 |
| 1.0 | 38 | 38 | 8 | 5 |
| 5.0 | 3 | 39 | 32 | 36 |

a. Values are means of two replicates, two trials per replicate.

IV. DISCUSSION AND CONCLUSIONS

It has often been reported that actively growing and metabolizing bacteria are more sensitive to adverse environmental factors than resting organisms.^{7/} For this reason, it was expected that the nonoxidizable sugars might stabilize *S. marcescens* against freeze drying and/or aerosolization, but the oxidizable sugars might not. The oxidizability of sugars and their stabilizing effects could not be correlated, however. Eventually it was observed that the penetrability classification of sugars was correlated with their ability to modify the survival of aerosolized or freeze-dried cells.

According to Rothstein,^{8/} there are at least three kinds of "spaces" available for dilution of solute in a preparation of packed cells:

(a) The first space is the interspace between packed cells. The theoretical value for close-packed spheres is 26 per cent. Experimental determinations made with macromolecules such as dextran and inulin have yielded values ranging from 6 to 25 per cent. As indicated previously, our experiments using dextran showed this space to equal 20 per cent of the cell-pack volume.

(b) The second space probably constitutes the cell-wall space. It lies outside the cytoplasmic membrane and is accessible to small molecules and ions, but not to macromolecules; values from 12 to 40 per cent or more have been reported. Since the MP sugars penetrated from 21 to 39 per cent of the cell volume, they were assumed to enter only this far into the cells.

(c) The third space lies within the cytoplasmic membrane. The FP sugars were believed capable of penetrating this membrane, an inference drawn from the high values of space available for dilution of these solutes.

According to this evaluation, the FP sugars penetrated the cell membrane; the MP sugars penetrated the cell wall but not the cell membrane. Also, the FP sugars stabilized freeze-dried cells, and the MP sugars stabilized aerosolized cells. These solute molecules could not have induced stability merely by their presence, however, because the effects of an MP or FP sugar were eliminated by a sufficient amount of a sugar of opposite penetrability. Therefore, it appeared that the sugars, separately or in combination, must have stabilized the cells indirectly through their effects on some other variable.

Sugars as a class are freely and rapidly soluble in water but are often difficult to crystallize out of solutions, especially in the presence of other solutes. Syrups are examples of impure concentrated sugar solutions that do not readily lose water by evaporation nor deposit solute by crystallization. The ability of sugar molecules to retain water molecules under such conditions has been termed "water-binding." It is hypothesized that

the stability of freeze-dried or aerosolized cells was changed in the presence of sugars because the water-binding properties of these solutes enabled them to change (a) the water content of the cells after they had equilibrated with the test stress, and/or (b) the rate of water loss by the cells during stress, and/or (c) the distribution of water between the inside and outside of the cells during and after stress. According to this hypothesis, freeze-dried cells required an optimum intracellular water content (IWC) for survival. In the absence of FP solutes, cells died during freeze drying because their IWC was reduced to an intolerably low level; one per cent of FP sugars raised the IWC to a level that enabled the cells to remain viable. In the presence of an excess of FP sugar such as five per cent sorbose, the IWC was insufficiently reduced to permit survival of freeze-dried cells. Five per cent sorbose was an effective stabilizer, however, in the presence of one per cent or more raffinose. Raffinose and the other MP sugars presumably could not penetrate the cytoplasmic membrane, and so enhance IWC and freeze drying survival levels. Raffinose, however, competitively reduced the amount of water that the FP sorbose retained within the cells, thus reducing the IWC to a level compatible with survival. By this mechanism the sugars were believed to alter the distribution of water between the inside and the outside of the cells.

The stability of aerosolized cells was increased by MP sugars but not by FP sugars. These effects might also have been induced through modifications of IWC by solutes, as shown in the following analysis: Cell suspensions containing MP sugars were converted to droplets upon aerosolization. As these droplets lost water during evaporative equilibration, the extracellular sugar solution might become concentrated enough to induce a plasmolytic dehydration of the cell. The water so removed from the cell would be lost promptly by evaporation, so that the MP sugar solution would serve as a water transport mechanism to accelerate the reduction of IWC. Thus, it appeared that rapid dehydration of aerosolized cells favored their survival, and unprotected cells probably died because their IWC remained too high for too long. If aerosol stability were induced by the high concentrations of MP sugars created by evaporation, then this hypothesis would predict that an increase in the amount of MP sugar in a suspension should not improve the aerosol stability of its cells. It was observed that ten per cent raffinose was not a more effective stabilizer than 1% raffinose.

Because of their penetrability, the FP sugars could not accelerate the rate at which IWC of aerosolized cells was reduced and so could not induce aerosol stability. However, these sugars could interfere with the dehydrating and stabilizing effects of the MP sugars, as shown when one per cent sorbose was added to one per cent raffinose. In the presence of a large excess of MP sugar such as ten per cent raffinose, the ability of FP sugars (one per cent sorbose) to elevate IWC and depress aerosol stability was overcome.

To recapitulate, FP sugars were assumed to elevate, and MP sugars to depress, the IWC of *S. marcescens*. Elevation of IWC promoted the survival of freeze-dried cells; depression of IWC favored the survival of aerosolized

cells. These experiments, however, gave no insight into the mechanisms through which unsuitable levels of IWC induced the death of aerosolized or freeze-dried cells.

Two other aspects of these data may be pointed out as follows:

(a) The penetrability of sugars into the cells was apparently a direct function of their molecular size and/or weight. All of the monosaccharides were FP; all of the di- and trisaccharides were MP. Some of the FP sugars, such as d-arabinose and sorbose, could not be oxidized by S. marcescens. These observations suggested that the FP sugar molecules might have entered the cells by simple diffusion through the cell membrane; MP molecules did not pass through because they were too large.

(b) According to the hypothesis presented earlier, every sugar should be capable of stabilizing cells against freeze drying or aerosolization but not against both stresses. Melibiose was the only sugar that did not enhance the stability of the cells toward either of the test stresses. Since melibiose was MP, its ineffectiveness during freeze drying was an anticipated result. Aerosolized cell suspensions supposedly containing melibiose, however, undoubtedly contained melibiose plus products of its oxidative degradation. This mixture, rather than melibiose alone, was ineffective as an aerosol stabilizer.

The oxidizability of melibiose, an MP sugar, was assumed to be due to the existence of constitutive cellular enzymes that rapidly hydrolyzed the disaccharide to its oxidizable components, galactose and glucose.

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