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Production of 2-Ketogluconic Acid by *Serratia marcescens*[1]

T. J. MISENHEIMER, R. F. ANDERSON,[2] A. A. LAGODA, AND D. D. TYLER

Northern Regional Research Laboratory, Northern Utilization Research and Development Division, U.S. Department of Agriculture, Peoria, Illinois

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ABSTRACT

MISENHEIMER, T. J. (Northern Regional Research Laboratory, Peoria, Ill.), R. F. ANDERSON, A. A. LAGODA, AND D. D. TYLER. Production of 2-ketogluconic acid by *Serratia marcescens*. Appl. Microbiol. **13**:393-396. 1965.—Production of 2-ketogluconic acid from glucose by fermentation with *Serratia marcescens* NRRL B-486 was studied in 20-liter stainless-steel fermentors. Conditions for 2-ketogluconic acid production included the following: glucose-salt medium, aeration rate of 0.75 volumes per volume per minute, agitation rate of 400 rev/min, temperature of 30 C, $CaCO_3$ to neutralize the acid formed, and a 5% (v/v) inoculum. Foaming was controlled with an antifoam agent added at intervals during the fermentation. When 120 g per liter of glucose were supplied, 95 to 100% yields of 2-ketogluconic acid were obtained in 16 hr. Larger amounts of glucose could be used in the fermentation provided that the carbohydrate was fed continuously. Continuous feeding of glucose to a total amount of 180 g per liter gave 95 to 100% yields of 2-ketogluconic acid in 24 hr; feeding glucose to a total amount of 240 g per liter gave 85 to 90% yields in 32 to 40 hr.

Commercially, 2-ketogluconic acid serves as an intermediate in the production of isoascorbic acid. 2-Ketogluconic acid is currently produced with strains of the genus *Pseudomonas* (Lockwood et al., 1942; Pfeifer et al., 1958). From 60 to 65% of theoretical yields of 2-ketogluconic acid are reached in 40 to 72 hr. Our investigations have shown that yields obtained with strains of *Serratia marcescens* are higher and that the fermentation time is shorter. In flask experiments, 10 strains of *S. marcescens* examined gave 80% or better of theoretical yield of 2-ketogluconic acid in 24 hr. This paper describes a fermentation process that gives 95 to 100% of theoretical yield of 2-ketogluconic acid in 16 hr with a simple and inexpensive medium.

MATERIALS AND METHODS

S. marcescens NRRL B-486 was chosen from the 10 strains examined because of the high yields of 2-ketogluconic acid produced in a short fermentation time. The organism was grown for 24 hr at 28 C on a slant of the following composition: Tryptone, 0.5%; yeast extract, 0.5%; glucose, 0.1%; K_2HPO_4, 0.1%; and agar, 2.0%—dissolved in tap water and with *p*H adjusted to 7.0 (Haynes, Wickerham, and Hesseltine, 1955). The organisms from one slant were suspended in 5.2 ml of sterile water. A 1-ml amount of this suspension was added to 100 ml of inoculum medium in a 300-ml Erlenmeyer flask. The inoculum medium, a modification of one used to produce large populations of *S. marcescens* by Benedict et al. (1957), was as follows: dried skim milk, 2.5%; Proto-peptone No. 159 (Wilson and Co., Chicago, Ill.), 1.5%; and glucose, 2%. The inoculum was incubated for 32 hr at 28 C on a rotary shaker at 200 rev/min.

Fermentations were carried out in 20-liter baffled stainless-steel fermentors (Dworschack, Lagoda, and Jackson, 1954) with 10 liters of production medium, similar to that used by Sharpe and Corman (1957), composed as follows: $(NH_4)_2SO_4$, 0.19%; Na_2SO_4, 0.05%; $MgSO_4$, 0.04%; KH_2PO_4, 0.5%; NaCl, 0.004%; $MnSO \cdot 4H_2O$, 0.004%; $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, 0.005%; glucose, 12%; and CaCO, 3%. All ingredients were sterilized together by injecting steam into both jacket and medium until 121 C was reached, then cooled to 30 C, and inoculated with 5% (v/v) of 32-hr inoculum. After inoculation, foaming was controlled with concentrated Dow Corning Antifoam B (Dow Chemical Co., Midland, Mich.), which was automatically added on demand (Dworschack et al., 1954).

Samples were taken at regular intervals, and paper chromatograms were run with the descending technique to determine the acids present. The solvent system employed was *n*-butanol–formic acid–water (4:1.5:1). After equilibration, the

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[2] Present address: Bioferm Division, International Minerals and Chemical Corp., Wasco, Calif.

papers were first irrigated with the solvent for 7 to 8 hr at 25 C, then air-dried, and sprayed with an alkaline solution of 0.04% bromcresol green in 95% ethanol (*p*H adjusted to 11.5 to 11.8 with NaOH). Acids appeared as yellow spots on a blue background. When dry, the papers were sprayed again with 0.1% orthophenylenediamine in 95% ethanol containing 1% HNO_3, and heated at 100 C for 4 to 5 min. The 2-ketogluconic acid appeared as a yellowish-green spot.

Total reducing substances were determined by the copper reduction method of Shaffer and Hartmann (1921). By the use of appropriate calculations, this value expressed the sum of the 2-ketogluconic acid and the glucose in the fermentation liquor. By use of paper chromatography, we established that no other reducing substances were present. The amount of 2-ketogluconic acid in the liquor was obtained by subtracting the weight of glucose, as determined by the glucose oxidase method of Huggett and Nixon (1957). Yields of 2-ketogluconic acid were calculated as follows:

$$\frac{\text{weight 2-ketogluconic acid}}{\text{weight glucose supplied}} \times \frac{180}{194} \times 100$$

= per cent yield of 2-ketogluconic acid

Consequently, yield is the percentage of 2-ketogluconic acid which theoretically could be formed by the complete conversion of the glucose supplied during fermentation.

Results and Discussion

Preliminary work was carried out in normal and indented 500-ml Erlenmeyer flasks containing 100 ml of the production medium. The flasks were incubated at 28 C on a rotary shaker at 200 rev/min. The effects of age and amount of inoculum, concentration of glucose, and variations in the salt composition of the medium were studied. Indented flasks were superior to unmodified flasks, indicating a high aeration requirement. It was also noted that better results were obtained if the entire medium was autoclaved together rather than sterilizing glucose, $CaCO_3$, and salts separately. These studies showed that 12% glucose was required to get maximal yields of 2-ketogluconic acid. The 3% inoculum proved optimal for the shake-flask experiments. 2-Ketogluconic acid yields in excess of 90% were routinely obtained in 24 to 32 hr.

From experiments with the modified Erlenmeyer flasks, it appeared that aeration was an important factor in both the yield of 2-ketogluconic acid and the rate of acid production. To study the effect of aeration and agitation more thoroughly, the fermentation was carried out in 20-liter baffled, stainless-steel fermentors fitted with impellers (11.4 cm) with vertical blades. Agitator speeds of 300 to 400 rev/min were necessary to get maximal yields (Fig. 1).

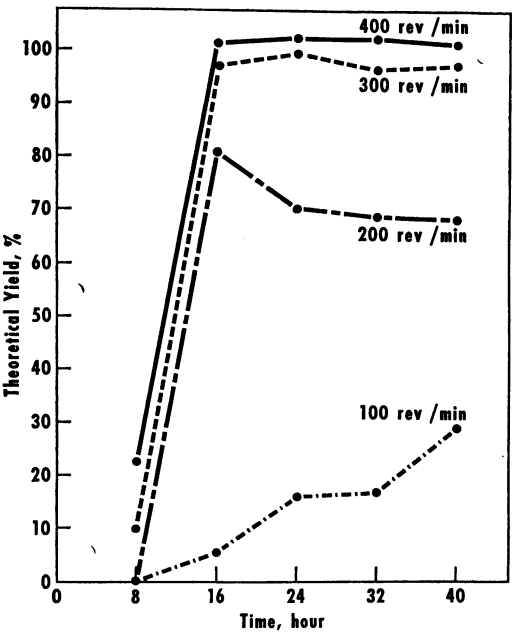

FIG. 1. *Effect of agitation on 2-ketogluconic acid production with an aeration rate of 0.75 volumes per minute and 12% glucose at 30 C.*

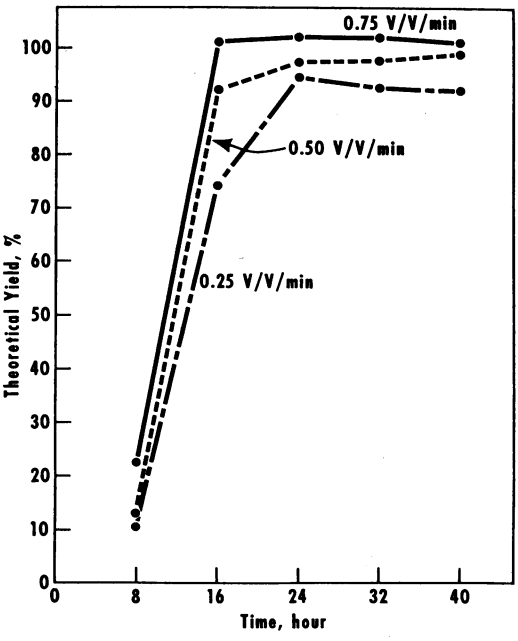

FIG. 2. *Effect of aeration on 2-ketogluconic acid production with agitation at 400 rev/min and 12% glucose at 30 C.*

The effect of aeration is shown in Fig. 2. Air volumes of 0.5 to 0.75 volumes per minute gave satisfactory yields in 16 hr. Excessive foaming

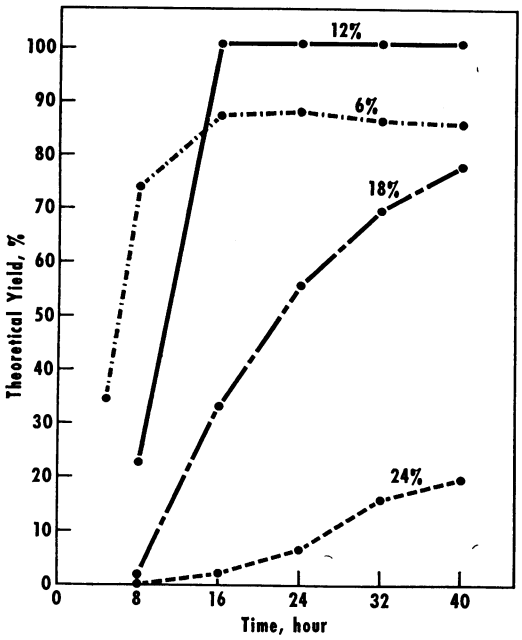

FIG. 3. *Effect of glucose concentration on 2-ketogluconic acid production with agitation at 400 rev/min, aeration rate of 0.75 volumes per minute, and temperature at 30 C.*

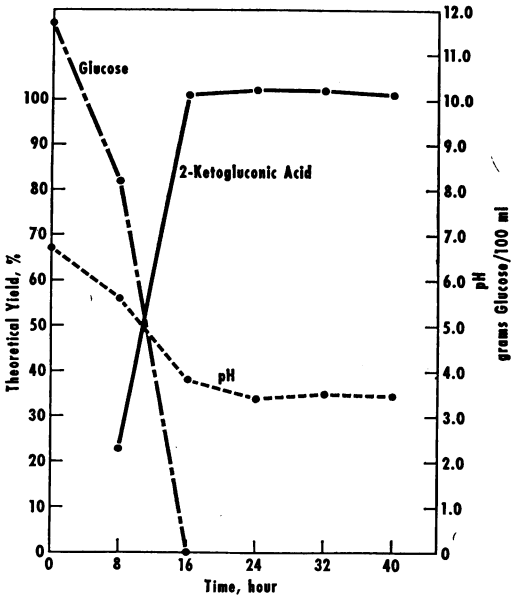

FIG. 4. *Typical 2-ketogluconic acid fermentation with 12% glucose at an agitation rate of 400 rev/min, aeration rate of 0.75 volumes per minute, and temperature at 30 C.*

occurred at the higher aeration rates. Often, considerable amounts of antifoam agent were needed to control the foam; this might not be a problem in a fermentor with more head space.

Figure 3 shows the effect of different concentrations of glucose in the production medium. At the lower concentrations glucose was utilized more rapidly in the early hours of the fermentation. When the initial concentration of the glucose was increased to 12%, theoretical yields of 2-ketogluconic acid were realized even though the fermentation was slower. Above 12% glucose, the rate of production of 2-ketogluconic acid was considerably reduced, and yields were diminished.

The 2-ketogluconic acid was neutralized as it was formed with the $CaCO_3$ present. A 1-g amount of $CaCO_3$ was supplied for every 4 g of glucose present in the medium.

In the early hours of a typical fermentation (Fig. 4), the sugar is utilized slowly and little 2-ketogluconic acid is formed. During this time the cells are multiplying rapidly. Plate counts, made on samples taken every 2 hr, showed a rapid increase in viable cells until 10 to 12 hr, at which time about 50% of the sugar had been used. The cell count then became constant until it began to drop in the later hours of the fermentation. 2-Ketogluconic acid was produced rapidly

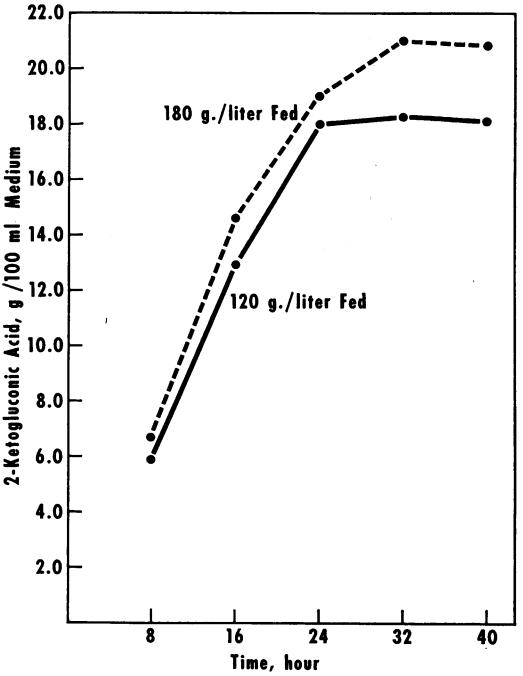

FIG. 5. *Effect of feeding glucose on 2-ketogluconic acid production with initial concentration of 6% glucose. Feeding started at 8 hr at 14 g per liter per hour, with an aeration rate of 0.75 volumes per volume per min, and agitation rate of 400 rev/min at 30 C.*

between 8 and 16 hr, and the remaining glucose was consumed.

The initial rate of 2-ketogluconic acid formation is much greater when the initial glucose concentration is below 12% (Fig. 3). Experiments were performed (Fig. 5) in which the initial sugar concentration was 6%. At the end of 8 hr, sugar was then fed continuously at the rate of 14 g per liter per hour. In addition to the original 60 g per liter, 120 g per liter were fed, and the yields of 2-ketogluconic acid were 90 to 100% of theory in 24 hr. Although the total amount of 2-ketogluconic acid formed per fermentor was increased, the overall rate of formation was not significantly altered. When the amount of glucose fed was increased to 180 g per liter for a total of 240 g per liter, the yield was below 90% of theory even though the fermentation was continued for 32 hr. Increasing the rate of feed did not alter yield.

An optimal concentration for quantitative conversion of glucose to 2-ketogluconate appears to be 12% glucose. With lower concentrations of glucose, 2-ketogluconate formed at a rapid rate, but the yield was less than theoretical. The lower yield may indicate that some of the glucose is used in alternate metabolic pathways, more nearly resembling the classical concept of *S. marcescens* metabolism, in which a variety of products are obtained, such as acetic acid, formic acid, CO_2, and ethanol. On the other hand, when the glucose concentration exceeds 12%, there is a definite inhibitory effect on the rate of 2-ketogluconate formation. Inhibition at the higher glucose levels appears to be due to a repressing effect by the glucose and not to excessive osmotic pressure, as was shown by adding up to 23% sucrose to a 12% glucose medium. Under these circumstances, the glucose utilization and 2-ketogluconate production parallels that in 12% glucose medium without added sucrose. The nature of this glucose effect is being further investigated.

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