Conditions for Production of 3-Ketomaltose from Agrobacterium tumefaciens

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Up to 39% yields of 3-ketomaltose were achieved in 18 to 22 hr when Agrobacterium tumefaciens NRRL B-36 was cultured at 25 to 28 C in a simple medium containing 4.0 to 8.0% maltose, 0.09% urea, 0.5% CaCO3, 0.6% KH2PO4, and 0.025% MgSO4.7H2O. For maximum production of 3-ketomaltose the culture had to be maintained approximately at pH 7.0.

Reportedly (1-3), strains of Agrobacterium tumefaciens and A. radiobacter can produce 4-(3-keto-α-d-glucosido)-d-glucose, designated as 3-ketomaltose (3-KM), from maltose. A variety of other 3-keto-d-glycosido-D-glycose (3-ketoglycosides) can be synthesized from corresponding disaccharides (7, 8). The 2,3-enediols of 3-ketoglycosides have the ability to deter oxidation and discoloration of aqueous foodstuffs (4). Furthermore, addition of 3-ketoglycosides to foodstuffs containing vitamin C retards the oxidation of the vitamin and thus permits the food to retain its vitamin activity. Fatty acid esters of the enediol can be used to prevent oxidation of oils and fats (4). Our interest in the practical use of maltose or its derivative prompted the study of conditions required for efficient production of 3-KM.

MATERIALS AND METHODS

Cultures. A. radiobacter NRRL B-164, B-165, B-181, and B-2398 and A. tumefaciens NRRL B-36, B-37, and B-38 were obtained from the Agricultural Research Service Culture Collection. The organisms were maintained on slants containing 2.0% glucose, 1.0% yeast extract, 2.0% CaCO3, and 2.0% agar in distilled water. All organisms were grown on the slants for 24 hr at 28 C and immediately used as a source of an inoculum or stored at 4 C.

Medium. The basal medium for the study of 3-KM production contained 4.0% maltose, 0.025% MgSO4.7H2O, 0.6% KH2PO4, and 0.2% (NH4)2SO4. The pH of the medium was adjusted with KOH to 7.0 before sterilization. This medium is a modification of one described by Fukui and Hochster (7). Maltose and the soluble salts were sterilized in separate solutions at 120 C for 15 min and aseptically mixed to produce the desired concentrations. CaCO3, when used, was autoclaved for 30 min and then dried in an oven at 110 C for 8 to 12 hr; the dry CaCO3 was added aseptically to sterilized media. Urea solutions were sterilized separately with a Seltz filter.

Inoculum preparation. Fresh cells from slants were transferred to 50 ml of medium in 300-ml Erlenmeyer flasks which were incubated for 24 hr at 28 C on a rotary shaker. Subsequently, 100 ml of medium in 500-ml Erlenmeyer flasks was inoculated with 2 ml of the inoculum culture. The inoculum was always developed in a medium with identical composition to that used for the production of 3-KM. In all experiments, flasks were incubated on a rotary shaker with a 2.25-inch diameter stroke at 200 rev/min.

Assay methods. Samples were removed periodically from the flasks and their pH values determined. The samples were centrifuged and the supernatant fluids were assayed for various sugars. 3-KM was determined by measuring the optical density at 340 nm of a sample diluted with 0.1 N NaOH (5). Under these alkaline conditions, no change in optical density at 340 nm was noted with glucose or maltose. Reports show that 3-ketoglucose (3-KG) does not absorb at 340 nm under alkaline conditions (5). 3-Ketoglucose was determined by measuring the optical density at 310 nm of a sample diluted with 0.2 M phosphate buffer, pH 7.0 (5). The sugar amounts were calculated by using the extinction coefficients of 5.5 × 103 M⁻¹ cm⁻¹ for 3-KM and 3.8 × 103 M⁻¹ cm⁻¹ for 3-KG as determined by Fukui and Hayano (5). Yields were calculated as the per cent by weight of initial maltose converted to 3-KM. All experiments were run in duplicate, and the average values were reported as the yield obtained.

Total reducing sugar, expressed as maltose, was measured with an autoanalyzer by using the potassium ferricyanide method (9). To obtain an approximate determination of residual maltose, a reducing sugar equivalent of 3-KM was subtracted from the total reducing sugar value. In terms of reducing power, 1.0 mg of 3-KM is equivalent to 2.1 mg of maltose. 3-Ketoglucose was not considered in this calculation because the small amount did not significantly affect the quantitative values for either residual maltose or 3-KM produced. Glucose was not detected with Glucostat reagent by the supplier's specifications.

Cell count. Optical density of cultures at 625 nm was taken after acidification to dissolve CaCO3, cen-
trifugation, and resuspension of cells in distilled water at the original volume. Cell numbers were estimated from a standard curve which plotted optical density against the number of cells counted with a Petroff-Hauser bacterial cell counter.

**Chemicals.** The chemicals used and suppliers were: maltose (Matheson, Coleman, and Bell, Cincinnati, Ohio), urea (Merck & Co., Inc., Rahway, N.J.; and Glucostat reagent (Worthington Biochemical Corp., Freehold, N.J.).

**RESULTS**

**Organism.** The ability of actively growing organisms to produce 3-ketoglycosides on a medium containing 2% agar, 0.1% yeast extract, and 1.0% of the appropriate glycoside can be detected by the appearance of a yellow zone around the colonies when the agar surface is flooded with Benedict's copper reagent (3). The yellow zone results from the reduction of the cupric ion by 3-ketoglycosides at room temperature. The ability of seven *Agrobacterium* cultures to produce 3-ketoglycosides is compared in Table 1. Both *A. radiobacter* and *A. tumefaciens* converted lactose to 3-ketolactose. However, *A. tumefaciens* NRRL B-36 and B-37 also gave positive effects with maltose. When tested in liquid medium, *A. tumefaciens* B-36 gave better yields of 3-KM; therefore, it was used for further studies.

**Phosphate buffer concentration.** Preliminary work showed that the buffering capacity of the basal medium was not sufficient to prevent the rapid decrease of the pH resulting from the growth of the organism. Therefore, the basal medium was modified by dissolving maltose, (NH₄)₂SO₄, and MgSO₄·7H₂O in KH₂PO₄-K₂HPO₄ buffer of varying concentrations, pH 7.0.

The results show that 3-KM production increases as the buffer concentration ranged from 0.025 to 0.25 M (Table 2). The enhancement of yield results mainly from the pH maintaining action of the buffer. For example, in the presence of 0.05, 0.10, and 0.25 M buffer, the rate of production of 3-KM was approximately equal for the first 24 hr, at which time the pH values ranged from 6.2 to 6.8. However, the yields obtained in the presence of 0.1 and 0.25 M buffer after 36 or 48 hr were substantially greater than that obtained in 0.05 M buffer (Table 2). The pH in the

**Table 1. Disaccharide oxidation by *Agrobacterium* radiobacter and *A. tumefaciens***

| Organism         | NRRL no. | Substrate<sup>a</sup> |
|------------------|----------|------------------------|
|                  |          | Lactose    | Maltose   |
| *Agrobacterium*  |          |            |            |
| radiobacter      | B-164    | +<sup>c</sup>  | -<sup>d</sup>  |
| *A. radiobacter* | B-165    | +  | -            |
| *A. radiobacter* | B-181    | +  | -            |
| *A. radiobacter* | B-2398   | -  | No growth   |
| *A. tumefaciens* | B-36     | +  | +            |
| *A. tumefaciens* | B-37     | +  | +            |
| *A. tumefaciens* | B-38     | -  | -            |

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<sup>b</sup> Agar plates containing 0.1% yeast extract, 2.0% agar, and 1.0% of either maltose or lactose were inoculated with a loopful of cells grown for 24 hr at 28 C on slants. The plates were incubated for 50 hr at 28 C. The agar surface of the plates were then flooded with Benedict's copper reagent and observed after 1 hr at room temperature.

<sup>c</sup> Reduction of copper reagent indicated by appearance of yellow zone around colonies.

<sup>d</sup> No reduction of copper reagent indicated by absence of yellow zone around colonies.

**Table 2. Effect of phosphate buffer concentration on 3-ketomaltose production by *Agrobacterium tumefaciens***

| Buffer, concn (M) | 12 HR | 24 HR | 36 HR | 48 HR |
|-------------------|-------|-------|-------|-------|
|                   | pH    | Yield (%) | pH    | Yield (%) | pH    | Yield (%) | pH    | Yield (%) |
| 0.025             | 6.5   | 1.0     | 5.4   | 5.1     | 4.8   | 5.5     | 4.8   | 5.7     |
| 0.050             | 6.7   | 1.1     | 6.2   | 7.5     | 5.7   | 12.9    | 5.1   | 14.1    |
| 0.10              | 6.8   | 1.3     | 6.5   | 9.3     | 6.3   | 21.5    | 6.2   | 21.8    |
| 0.25              | 6.9   | 1.0     | 6.8   | 7.7     | 6.7   | 18.8    | 6.6   | 21.8    |
| 0.50              | 6.9   | 0.4     | 6.9   | 1.1     | 6.8   | 2.9     | 6.8   | 10.5    |
| 0.75              | Poor growth | Poor growth | Poor growth | Poor growth | Poor growth | Poor growth | Poor growth | Poor growth |
| 1.0               | Poor growth | Poor growth | Poor growth | Poor growth | Poor growth | Poor growth | Poor growth | Poor growth |

<sup>a</sup> Production of 3-ketomaltose was carried out in 100 ml of medium per 500-ml Erlenmeyer flask containing 4.0% maltose, 0.2% (NH₄)₂SO₄, 0.025% MgSO₄·7H₂O, and KH₂PO₄-K₂HPO₄ buffer at specified concentrations at initial pH 7.0. Incubation was at 28 C on a rotary shaker. Production flasks were inoculated with 2 ml of fresh cells grown in 50 ml of the same medium in 300-ml Erlenmeyer flasks incubated for 24 hr at 28 C.
presence of 0.05 m buffer at 36 and 48 hr had decreased to 5.7 and 5.1, respectively (Table 2). Visual examination showed that poor growth occurred when the buffer concentrations were greater than 0.25 m. Consequently, the yields of 3-KM were also low.

**Effect of pH.** Fukui and Hochster (6) showed that pH exerts a marked influence on the transport of sucrose by resting cells of *A. tumefaciens*. The gross effect is an increase or decrease of 3-ketosucrose synthesis with variation of the pH. Experiments were therefore carried out to study the effect of pH on 3-KM production by actively growing bacteria.

To study the effect of initial pH on 3-KM production, the basal medium components were dissolved in 0.25 m KH$_2$PO$_4$-K$_3$HPO$_4$ buffer at the desired initial pH. The initial pH markedly influences 3-KM production (Table 3). When the initial pH was 6.0 or less, the yield obtained at 48 hr was 7.8% at most. However, yields of 15.5 to 27% in 48 hr were realized when the initial pH ranged from 6.5 to 7.5. Therefore, in subsequent experiments, the medium was adjusted initially to pH 7.0.

Addition of CaCO$_3$ is an alternate method for pH control during the fermentation. To study this control method, the basal medium was supplemented with 0.1% to 1.0% CaCO$_3$. At least 0.5% CaCO$_3$ is required to maintain medium pH above 6.0.

As Fig. 1 shows, 3-KM yields reached a maximum of 27% when 0.5% CaCO$_3$ was used in the medium.

**KH$_2$PO$_4$-CaCO$_3$ effect.** Preliminary studies indicated that phosphate concentration affected the fermentation in addition to controlling the pH; therefore, we studied the combined effect of KH$_2$PO$_4$ concentration with 0.5% CaCO$_3$ in the basal medium. With 0.6 and 0.9% KH$_2$PO$_4$, approximately 30% yield was realized in 32 hr (Fig. 2). Comparable yields can be obtained at the 1.2% phosphate concentration, but a longer time is generally required. Therefore, in all subsequent experiments, the basal medium was modified to contain 0.5% CaCO$_3$.

**Nitrogen source.** Studies revealed that varying (NH$_4$)$_2$SO$_4$ concentration above or below 0.2% apparently caused no significant increase in 3-KM production. Therefore, sodium nitrate, peptone, yeast extract, urea, (NH$_4$)H$_2$PO$_4$, and Casamino Acids were tested for their effect on

**TABLE 3. Influence of initial pH on 3-ketomaltose yields**

| pH   | Initial | Final | 22 Hr (% yield) | 48 Hr (% yield) |
|------|---------|-------|----------------|-----------------|
| 5.0  | 5.1     | <1.0  | <1.0           |                 |
| 5.5  | 5.2     | 1.2   | 2.1            |                 |
| 6.0  | 5.7     | 3.1   | 7.8            |                 |
| 6.5  | 6.2     | 4.7   | 15.5           |                 |
| 7.0  | 6.6     | 6.4   | 23.0           |                 |
| 7.5  | 6.9     | 7.0   | 27.5           |                 |

* Culture conditions were similar to those described in Table 2 except the phosphate buffer concentration was 0.25 m at each pH level.
TABLE 4. Effect of nitrogen source and concentration on 3-ketomaltose production

| Nitrogen concen (%) | Nitrogen source | 20 Hr | 24 Hr | 28 Hr |
|---------------------|-----------------|-------|-------|-------|
|                     | pH              | Yield (%) | pH | Yield (%) | pH | Yield (%) |
| 0.021               | (NH₄)₂SO₄       | 7.0    | 23.0 | 7.0 | 25.8 | 7.0 | 24.5 |
| 0.021               | Urea            | 7.2    | 26.2 | 7.2 | 27.9 | 7.1 | 25.4 |
| 0.021               | Casamino Acids  | 7.2    | 10.7 | 7.3 | 12.3 | 7.3 | 14.7 |
| 0.021               | (NH₄)₂H₂PO₄     | 7.1    | 20.5 | 7.0 | 22.5 | 7.0 | 23.0 |
| 0.042               | (NH₄)₂SO₄       | 6.8    | 20.0 | 6.8 | 25.0 | 6.8 | 26.2 |
| 0.042               | Urea            | 7.2    | 36.0 | 7.2 | 32.8 | 7.2 | 26.2 |
| 0.042               | Casamino Acids  | 7.2    | 16.4 | 7.3 | 23.8 | 7.3 | 25.8 |
| 0.042               | (NH₄)H₂PO₄      | 6.7    | 20.5 | 6.8 | 27.0 | 6.8 | 28.7 |
| 0.084               | (NH₄)₂SO₄       | 6.4    | 16.4 | 6.3 | 18.5 | 6.1 | 18.8 |
| 0.084               | Urea            | 7.4    | 31.0 | 7.2 | 30.8 | 7.1 | 27.0 |
| 0.084               | Casamino Acids  | 7.2    | 17.2 | 7.3 | 25.0 | 7.2 | 31.5 |
| 0.084               | (NH₄)H₂PO₄      | 6.4    | 18.5 | 6.1 | 24.2 | 6.0 | 26.2 |

* One hundred milliliters of media in 500-ml Erlenmeyer flasks contained 4.0% maltose, 0.6% KH₂PO₄, 0.025% MgSO₄·7H₂O, 0.5% CaCO₃, and the specified nitrogen source. Protocol for inoculation and incubation were identical to that described in Table 2.

the fermentation. The concentration of each nitrogen source added to the modified basal medium was such that the nitrogen level added would be equivalent to that present in 0.2% (NH₄)₂SO₄ (0.042% nitrogen). Casamino Acids, (NH₄)₂SO₄, urea, and (NH₄)₂H₂PO₄ were selected from this initial study for further experimentation because they all supported about 20 to 30% conversion of maltose to 3-KM in 24 hr.

Media for this study contained each nitrogen source at such level that the nitrogen concentrations were 0.021, 0.042, and 0.084%. On the basis of data in Table 4, urea is superior to the other nitrogen sources tried. Conversion of 36% of the maltose to 3-KM occurred in 20 hr with 0.09% urea (0.042% nitrogen). The next effective nitrogen source was Casamino Acids. At 0.85% Casamino Acids (0.084% nitrogen), the yield was 31.5% in 28 hr. Therefore, not only are yields better with urea than with the other nitrogen sources, but the time for obtaining peak production is less. Another advantage with urea is that the pH remains between 7.0 and 7.5 throughout the fermentation, a range which also contributes to good 3-KM production (Table 4).

**Temperature.** The effect of temperature on 3-KM synthesis was studied in a medium containing 4.0% maltose, 0.6% KH₂PO₄, 0.5% CaCO₃, 0.025% MgSO₄·7H₂O, and 0.09% urea. Yields of 30% 3-KM were realized in 20 to 24 hr when the fermentation was carried out at 25 to 28 C (Table 5). At 35 C a yield of only 11%, 3-KM results in 30 hr.

**Maltose concentration.** The effect of initial maltose levels at 4.0, 8.0, and 12.0% on 3-KM production was examined in a medium containing 0.6% KH₂PO₄, 0.025% MgSO₄·7H₂O, 0.09% urea, and 0.5% CaCO₃. Figure 3 shows that a 35% yield occurs in 28 hr when the initial maltose level is 8.0%. In 20 hr, however, the yield of 3-KM was 32% when the initial maltose concentration was 4.0%. Increasing the initial concentration to 12.0% slows fermentation and produces a 28% yield in 40 hr. In the presence of 0.045 or 0.18% urea, although the fermentation pattern was similar to that from 0.09% urea, the yields decreased. The respective yields with 4.0, 8.0, and 12.0% maltose in the presence of 0.045% urea were 21.6, 16.5, and 7.9%. In the presence of 0.18% urea, the respective yields for the same series of maltose concentrations were 25.5, 24.3, and 15.3%..

**Other factors.** Optimum production of 3-KM yield occurs if the seed culture is between 18 and 32 hr old and if the volume of inoculum is between 2 and 10%. Use of cultures less than
18 hr or more than 32 hr old results in decreased 3-KM yields. If inoculum volume is less than 2%, the rate of fermentation is reduced, and the final yield is decreased. For most of this work, a 2% inoculum 20 to 24 hr old was used.

**Fermentation pattern.** Figure 4 presents a typical fermentation observed in medium containing 4.0% maltose, 0.6% KH₂PO₄, 0.025% MgSO₄·7H₂O, 0.09% urea, and 0.5% CaCO₃. After an initial lag phase of 4 to 8 hr, both cell population and 3-KM formation increase rapidly to a maximum at 18 to 20 hr. At this time the amount of 3-KM produced is 15.6 mg/ml which is equivalent to 39% yield. Between 4 and 18 hr almost all the maltose is consumed. After 18 to 20 hr, 3-KM formation ceases and, subsequently, 3-KM gradually disappears. Cell proliferation also stops. A low production of 3-KG occurs throughout the course of the fermentation. The pH increases slightly during the lag phase, decreases to approximately the initial pH of 7.0 during the maximum 3-KM formation period, and then remains relatively constant until the fermentation ends. The total amount of reducing substances decreases gradually during fermentation.

**DISCUSSION**

Our study establishes cultural conditions for fermentations that gave yields of 36 to 39% conversion of maltose to 3-KM. Yields were consistently good when pH of the medium was maintained near neutrality. At pH of 7.0 to 7.5, the sugars are probably transported across the cell membrane most rapidly (6). Also, 3-ketoglycosides are unstable at a pH of less than 6.0 or higher than 7.5 (7, 8). We found that pH was adequately maintained at about 7.0 in a medium which contained 0.5% CaCO₃, 0.6% KH₂PO₄, and 0.09% urea. In addition to helping maintain pH, urea enhances the production of 3-KM in terms of yield and fermentation time. Yields of 36 to 39% were obtained in 20 hr. When other nitrogen sources were used, yields ranged from 25 to 30% after 28 hr. Urea is also preferred because it is at least twice as productive per unit weight as the other nitrogen sources.

It is interesting to note that the maximum yields obtained are transitory. When urea is the nitrogen source, yields peak at about 20 hr and decrease thereafter (Table 4 and Fig. 4). Yields reach maximum values later with other nitrogen sources, e.g., (NH₄)₂SO₄, and subsequently drop (Figs. 1 and 2). Other workers have also noted the occurrence of a decrease in yields of 3-ketoglycosides after the attainment of maximum production (1, 6, 7). Consumption by the organism is probably the main cause of the disappearance of the 3-KM.

Work with variations in maltose concentration showed that at 12 hr the amount of 3-KM formed decreased as maltose level increased. The rate of 3-KM formation was inversely related to maltose concentration. However, after 12 hr the rates of 3-KM formation were approximately the same at the three concentrations. With 4.0% maltose, the rate was maintained to 16 hr, with 8% maltose to 28 hr, and with 12.0% maltose to approximately 28 hr. Thereafter, the rate is
reduced but is maintained to 36 hr. The maintenance of similar rates over extended periods as initial maltose concentration increased accounts for the different incubation times required for peak formation of 3-KM (Fig. 3). In terms of 3-KM yield, the respective maxima from 4.0, 8.0, and 12.0% maltose were 32, 35, and 28%.

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