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# Biosynthesis of D-Mannitol from D-Glucose by *Aspergillus candidus*

K. L. SMILEY, M. C. CADMUS, and PATRICIA LIEPINS
*Northern Regional Research Laboratory,**
*Peoria, Illinois 61604*

## Summary

Mannitol has long been known as a product of glucose metabolism by some strains of *Aspergillus*. Apparently no concerted effort has been made to develop a practical fermentation process to make mannitol. Work at the Northern Laboratory has shown that nearly all strains of white *Aspergillus* produce significant amounts of mannitol; many strains of black *Aspergillus* also have this characteristic. *Aspergillus candidus* NRRL 305 is an exceptionally good mannitol producer. Studies on a fermentation process were conducted in 20-l. stainless steel fermentors, without baffles. Czapek-Dox medium, modified by addition of corn meal, yeast extract, and enzymatically hydrolyzed casein was the most satisfactory medium tested. Suitable increments of glucose were fed daily to the fermentors. The duration of the fermentation was from 10 to 16 days. The effects of agitation, aeration, temperature, and pH of the medium were studied. Under optimal conditions yields of mannitol approached 50% of the glucose consumed.

## INTRODUCTION

During the isolation of enzymes from concentrated crude culture filtrates of *Aspergillus niger* NRRL 337, large amounts of a crystalline product formed upon the addition of alcohol. The crystals were identified as D-mannitol, which will hereafter be called "mannitol." An examination of the literature showed that Birkenshaw et al.[1] had studied the formation of mannitol from glucose by various species of *Aspergillus*. They obtained yields as high as 50% of the glucose utilized, but the fermentations were extremely slow, requiring from 1 to 2 months. They also showed that in order to achieve such

* A laboratory of the Northern Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture.

yields they had to restrict the aeration to a few minutes each day. This restriction would seem reasonable since mannitol is a reduction product of glucose fermentation. Mannitol has also been reported by Yamasaki and Shimomura[2] to be formed by molds of the *A. glacus* group from glycerol. Apparently there has been no work done on the formation of this polyol by the *Aspergillus* since their studies some 30 years ago. This paper reports on some of the factors influencing mannitol formation by *A. candidus*.

## MATERIALS AND METHODS

### Cultures

Approximately 50 *Aspergillus* strains were screened for their ability to produce mannitol. All cultures were provided by the ARS Culture Collection maintained at the Northern Regional Research Laboratory. *A. candidus* NRRL 305 and NRRL 3248 were selected for further study.

### Media

Czapek-Dox medium, prepared according to the directions of Thom and Raper,[3] was modified by the substitution of glucose for sucrose and by the addition of whole ground corn and yeast extract. Inoculum medium was 5% ground corn containing 0.5% yeast extract.

### Fermentors

Shake flasks consisted of 150 ml. of medium in 500-ml. Erlenmeyer flasks. Some trials were also made in Fernbach flasks with variable amounts of medium. The flasks were shaken on a Gump rotary shaker running at 200 rpm. Incubation temperature was 28°C.

Fermentation was also conducted in 20-l. stainless-steel fermentors containing 9 or 10 l. of medium. The fermentors were equipped with variable-speed agitators, air spargers, and the usual accessories for temperature control.

### Analytical Methods

Mannitol was assayed by gas–liquid chromatography of trimethylsilyl derivatives, prepared according to the method of Sweely et al.,[4]

on an F&M* Model 720 gas chromatograph equipped with an integrator. D-Arabitol was used as an internal standard. Assay results were accurate within ±3%. Mannitol yields were based on the sugar actually consumed. Quantitative recovery of mannitol was accomplished by addition of six volumes of ethanol to a crude culture filtrate. Crystallization was allowed to proceed overnight in the cold. The crystals were filtered, washed, redissolved, and recrystallized. After the crystals were dried, the melting point was 167.5–168.5°C. No melting point depression was observed when the crystals were mixed with authentic mannitol.

Glucose was determined on an Autoanalyzer by adaption of the method described by Hoffman[5] utilizing reduction of alkaline ferricyanide.

## RESULTS

### Medium

Birkenshaw et al.[1] used Czapek-Dox medium containing 5% D-glucose. With limited aeration and extended incubation times they obtained yields of around 50% mannitol from D-glucose. To decrease the fermentation period, attempts were made to produce mannitol in shaker flasks. It soon became evident that mannitol was rapidly formed in the first 2–3 days and was then quickly metabolized by the mold. The mold also tended to grow in the form of large pellets under these conditions. To get uniform growth, the medium was supplemented with ground corn which effectively kept the mycelium dispersed. To prevent mannitol from being metabolized, glucose was fed to the flasks daily. The most effective medium consisted of 3.5% ground whole corn, the salts of Czapek's medium, 1% Difco yeast extract, and glucose added batchwise at the rate of 3 g./100 ml. at 24 and 48 hr. and 1 g./100 ml. daily thereafter. The fermentation then proceeded for 10 days or more without loss of mannitol.

The individual salts in Czapek's medium were tested for their effect on mannitol formation. The results in Table I show that in-

* The mention of trade products or firm names does not constitute an endorsement by the U.S. Department of Agriculture over other products or firm names not mentioned.

TABLE I
Effect of Salts on Mannitol Formation by *Aspergillus candidus* NRRL 305[a]

| $NaNO_3$, % | $K_2HPO_4$, % | $MgSO_4 \cdot 7H_2O$, % | KCl, % | $FeSO_4$, % | Yield D-mannitol at 10 days, % |
|---|---|---|---|---|---|
| — | 0.1 | 0.05 | 0.05 | 0.001 | 16.1 |
| 0.2 | 0.1 | 0.05 | 0.05 | 0.001 | 26.2 |
| 0.4 | 0.1 | 0.05 | 0.05 | 0.001 | 31.9 |
| 0.2 | ... | 0.05 | 0.05 | 0.001 | 26.9 |
| 0.2 | 0.5 | 0.05 | 0.05 | 0.001 | 31.3 |
| 0.2 | 0.1 | ... | 0.05 | 0.001 | 26.1 |
| 0.2 | 0.1 | 0.15 | 0.05 | 0.001 | 23.3 |
| 0.2 | 0.1 | 0.05 | ... | 0.001 | 24.2 |
| 0.2 | 0.1 | 0.05 | 0.15 | 0.001 | 30.1 |
| 0.2 | 0.1 | 0.05 | 0.05 | ... | 26.3 |
| 0.2 | 0.1 | 0.05 | 0.05 | 0.005 | 27.0 |
| ... | ... | ... | ... | ... | 14.6 |

[a] All flasks contained 3.3% corn meal and 1% yeast extract, and glucose was fed daily (14 g. total).

creasing $NaNO_3$, KCl, and $K_2HPO_4$ slightly increases mannitol yield. Deletion of $NaNO_3$ causes a sharp yield loss. Individual deletions

TABLE II
Suitability of Different Organic Nitrogen Sources for Mannitol Production by *Aspergillus candidus* NRRL 305[a]

| Nitrogen source, 1% | 10-day yield of D-mannitol, % of theory |
|---|---|
| Yeast extract | 28.7 |
| Tryptone | 24.1 |
| Peptone | 19.6 |
| Malt extract | 9.2 |
| Amber EHC | 18.6 |
| Amber BYF | 19.0 |
| Amber HSP | 21.8 |
| Vico D-200 | 19.6 |
| Vico D-300 | 12.9 |
| Edamin | 12.6 |

[a] All flasks contained 3.3% corn meal and Czapek's salts, and glucose was fed daily.

of the other salts have little effect, but if all salts are deleted, the yield is reduced, although not significantly below that found with omission of $NaNO_3$ alone. Increasing $NaNO_3$ beyond 0.4% also lowers yields.

A number of organic nitrogen sources were tested for their suitability for mannitol formation by NRRL 305 (Table II). Only Difco tryptone approaches yeast extract as a suitable nitrogen source. It was noticed that flasks containing Amber EHC did not foam as did other nitrogen sources. Subsequently it was found that 0.1% Amber EHC would control foaming, and it was routinely added to the medium.

Table III shows that yeast extract is required at levels of around

TABLE III
Effect of Yeast Extract Concentration on Mannitol Formation by NRRL 3248[a]

| Yeast, extract, % | D-Mannitol in culture 14 days, % | Physiological yield, % |
|---|---|---|
| 0.25 | 3.18 | 15.4 |
| 0.75 | 6.24 | 25.3 |
| 1.00 | 6.64 | 26.6 |
| 1.25 | 6.96 | 27.8 |

[a] Conditions same as shown in Table II.

1% to get maximum conversion of D-glucose to mannitol. Thus far, Difco yeast extract has been the best organic nitrogen source tested. Undoubtedly, a more economical nitrogen source could be found to replace yeast extract.

## Aeration

The work of Birkenshaw et al.[1] indicated that limited aeration was required for maximum yields of mannitol. Since our work was conducted in shaker flasks where aeration is more efficient than in stationary ones, an experiment was run to see what the effect of volume of medium in the flasks would have on mannitol formation. Fernbach flasks were used containing 500, 750, 1000, and 1250 ml. of medium and were fed daily in the manner described. Figure 1

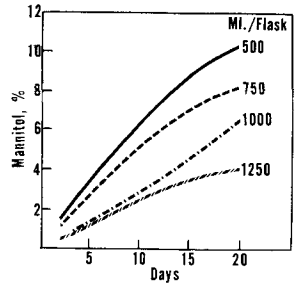


Fig. 1. Effect of volume of medium in 2800-ml. Fernbach flasks on D-mannitol production by *Aspergillus candidus* NRRL 305.

indicates that the most highly aerating conditions gave the best yields of mannitol.

## Initial pH

The pH of the medium is fairly critical. Figure 2 shows the effect

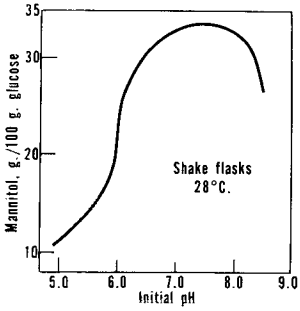


Fig. 2. Effect of initial pH of the medium on D-mannitol production by *A. candidus* NRRL 3248.

of initial pH on mannitol yields by NRRL 3248. Similar results occurred with NRRL 305. The pH must be around neutral for maximum yields. The effect of pH is early in the fermentation, since at 10 days or longer the pH of all the flasks are about the same and are around 6.0. No attempt was made to adjust pH during fermentation. On the mediums used, apparently, the organism does not produce much acid. Paper chromatographic analyses of culture

filtrates for organic acids showed only small amounts of malic acid with traces of other organic acids.

## Inoculum

Inoculum was prepared by transferring a loop of spores from a stock slant of the culture to a 500-ml. flask containing 100 ml. of medium consisting of 5% ground whole corn plus 0.5% yeast extract. The flask was incubated on a rotary shaker at 28°C. for 2 days. A 10% transfer to another flask of the same medium was then made and incubated similarly. The second flask was used as inoculum for the experiment. Experience showed that about 10% inoculum was optimum. No lag in mannitol formation was apparent when this procedure was used.

## Twenty-Liter Fermentor Trials

The effects of temperature, aeration, and agitation can be measured more precisely in stirred vessels. For this purpose 20-l. stainless steel fermentors equipped with individual stirring devices and temperature controllers were employed. Each vessel had an operating capacity of 10 l. and was stirred with a 4-in. impeller. Air was admitted through a sparge pipe. The vessels are essentially the same as those described by Dworschack et al.[6]

*A. candidus* NRRL 3248 showed an optimum temperature of 31°C. For the first 9 days there was no difference in rate of mannitol formation at 31 or 34°C., and 0.67 g. mannitol/100 ml./day were produced. However, in the last 4 days 0.92 g./100 ml./day were produced at 31°C.; at 34°C. the rate dropped to 0.3 g./100 ml./day. With

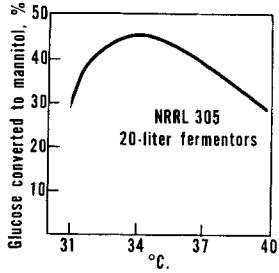


Fig. 3. The influence of temperature on D-mannitol production by *A. candidus* NRRL 305. The run was 13 days in 20-l. fermentors.

NRRL 305 there was no appreciable difference in rate over the entire fermentation time of 13 days. At 34°C. the average production rate was 0.64 g./100 ml./day, whereas at 31°C. the rate was 0.4 g./100 ml./day. Figure 3 shows the effect of temperature for NRRL 305.

Optimum agitation speed appears to be about 500 rpm. Speeds of 550 rpm significantly lower the yield though the glucose is totally utilized. Agitation speeds below 500 rpm reduce the yield of mannitol slightly (Fig. 4).

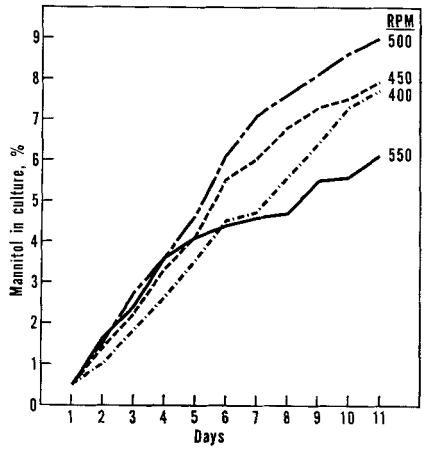


Fig. 4. Effect of agitation on D-mannitol production by *A. candidus* NRRL 305 in 20-l. fermentors.

Air rates from 0.25 to 1.5 v/v were tried. Yields were highest and comparable at 0.5 and 1.0 v/v. Higher flow rates caused excessive evaporation in the equipment used.

## DISCUSSION

The process described here for the production of mannitol by *A. candidus* contrasts rather sharply with the process described by Birkenshaw et al.[1] In the present process, air requirements were found to be comparable with other aerobic fermentation processes, in spite of the reduced nature of the product, mannitol. Their process called for extremely limited aeration. They used closed flasks and aerated with a stream of air for 30 min. daily. If flasks

were allowed to incubate with cotton plug closures, they noticed a drastic reduction in mannitol yield. No attempt was made in our work to duplicate their procedure, but it was noticed that at 5% initial sugar levels mannitol was formed and then rapidly disappeared. Apparently, no analyses for mannitol were made in the first few days by the English workers.

The presence of glucose in the medium is essential to prevent the metabolism of mannitol. This need was conveniently fulfilled by daily addition of glucose. Continuous feeding of glucose should also serve this purpose. Figure 5 illustrates the effect of glucose

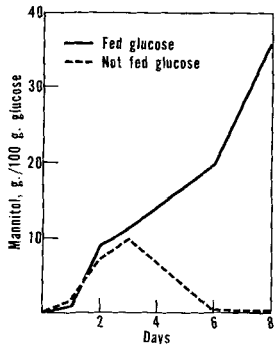


Fig. 5. Influence of glucose feeding on D-mannitol formation by *A. candidus* NRRL 305. Glucose was fed at the rate of 3%/day for the first 2 days and then 1%/day for the rest of the time.

feeding on mannitol accumulation. The relative efficiency of mannitol production is about the same for the first 3 days whether or not glucose is fed. After 3 days mannitol is rapidly consumed if glucose is not supplied.

The course of a typical mannitol fermentation is depicted in Figure 6. For about 12 days, mannitol formation is steady and the ratio of mannitol formed to glucose consumed increases during this time. The pH, after an initial drop from 7.0 to 6.0, remains steady throughout the fermentation at about 5.5 to 6.0. No attempt was made to control pH. The efficiency of the conversion of glucose to mannitol also rises as the fermentation progresses. Table IV shows the effect of age of the fermentation on the efficiency with which mannitol is formed from glucose. The lower conversion during the first 3 days is probably because much of the glucose is used for growth.

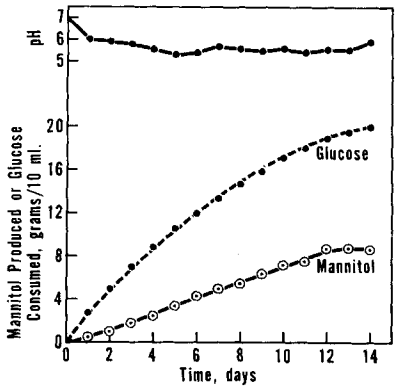


Fig. 6. Course of a typical mannitol fermentation by *A. candidus* NRRL 305.

TABLE IV
Effect of Age of Fermentation on the Ratio of Mannitol Production from Glucose by *A. Candidus* NRRL 305

| Time interval, days | Glucose utilized/ 100 ml., g. | Mannitol formed/ 100 ml., g. | Yield of mannitol from glucose, % |
|---|---|---|---|
| 1–3 | 7.0 | 1.8 | 26 |
| 4–6 | 5.0 | 2.2 | 44 |
| 7–9 | 3.8 | 2.2 | 53 |
| 10–12 | 3.2 | 2.2 | 69 |

The reason for the abrupt cessation of mannitol formation after 12 days is not known but is possibly caused by general autolysis of the mycelium.

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