Reduction of Acetoin to 2,3-Butanediol in 
Klebsiella pneumoniae: A New Model

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Summary
Fermentation of xylose by Klebsiella pneumoniae (ATCC 8724, formerly known as Aerobacter aegengenes) carried out in our laboratory yields 2,3-butanediol as the major product. Experimental data obtained in this work cannot be explained by the model presently in the literature for the formation of 2,3-butanediol isomers from acetoin isomers. A new model is proposed with the existence of two acetoin reductases and an acetoin racemase. The two reductases were separated and their stereospecificity determined. Extension of the model of other microorganisms is discussed.

INTRODUCTION
A viscous liquid, 2,3-butanediol (2,3-BD) can be produced by fermentation of glucose1 or xylose.2,3 It has a high combustion value and, more importantly, can be converted into 1,3-butadiene either directly or via its diaceto derivative.1 The production of 2,3-butanediol from pentoses is of current interest since pentoses are major products that arise from biomass hemicellulose.

Figure 1 summarizes the pathway for formation of 2,3-BD from α-xylose (pentose) or α-glucose. In 1952, Juni4 showed that Klebsiella pneumoniae forms acetoin (3-hydroxybutanone) from pyruvate by the action of two enzymes: 1) acetolactate-synthase (E.C. 4.1.3.18), which catalyzes the condensation of two pyruvate molecules and a single decarboxylation to yield acetolactate, and 2) acetolactate decarboxylase (E.C. 4.1.1.5), which catalyzes the decarboxylation of acetolactate to acetoin (Fig. 2). The acetolactate decarboxylase is specific for dextrorotatory isomer of acetolactate and the reaction product is the α-lactoin. Both acetolactate synthase and acetolactate decarboxylase of K. pneumoniae have been partially purified and characterized.5,6

The acetoin produced can either be oxidized to diacetyl by O2 present in the fermentation medium or enzymatically reduced to 2,3-BD. Reduction requires NADH as cofactors (Fig. 2); 2,3-BD exists in three isomeric forms: α (−), β (+), and meso. All three isomers occur in nature, and the isomeric composition of the 2,3-BD produced varies with the microorganism used in the fermentation.1,7,8

In 1960, Taylor and Juni9 proposed a model for the formation of 2,3-BD
steroisomers from acetoin (Fig. 3), based on the observed optical rotation of acetoin produced from pyruvate, the composition of 2,3-BD stereoisomers formed in the fermentation, and the rates of oxidation of 2,3-BD stereoisomers. The model proposed the existence of three enzymes, acetoin racemase (E.C. 5.1.2.4), \( (+) \) 2,3-BD dehydrogenase (E.C. 1.1.1.76), and a \( (-) \) 2,3-BD dehydrogenase (E.C. 1.1.1.4). The dehydrogenases were said to be nonspecific with respect to acetoin stereoisomers. That is, they would accept either acetoin isomer as substrate, but the stereochemistry of the reaction product would still depend on the acetoin isomer reduced. For example, the \( (+) \) de-
HYDROGENASE WOULD REDUCE L(+) ACETOIN TO L(+) 2,3-BD AND L(-) ACETOIN TO MESO 2,3-BD.

FOR K. PNEUMONIAE, TAYLOR AND JUNI PROPOSED THE PRESENCE OF ACETOIN RACEMASE AND L(+) 2,3-BD DEHYDROGENASE. WHILE THEY WERE UNABLE TO DOCUMENT THE PRESENCE OF ACETOIN RACEMASE IN ACETONE-DRIED PREPARATIONS OF K. PNEUMONIAE, THEY SPECULATED THAT CELL EXTRACTS PREPARED BY OTHER PROCEDURES WOULD SUBSEQUENTLY BE SHOWN TO CONTAIN AN ACETOIN RACEMASE ACTIVITY. NONE OF THE ENZYMES DESCRIBED BY TAYLOR AND JUNI HAVE BEEN PURIFIED AND CHARACTERIZED.

THE PURIFICATION AND REPORTED CHARACTERIZATION OF A DIACETYL (ACETOIN) REDUCTASE (ACETOIN NAD+ OXIDOREDUCTASE, E.C. 1.1.1.5) FROM K. PNEUMONIAE10-11 DOES NOT CHARACTERIZE THIS ENZYME WITH RESPECT TO THE STEREOSCOMPOSITION OF THE SUBSTRATE AND THE PRODUCT.

MATERIALS AND METHODS

GROWTH OF CELLS AND PREPARATION OF CELL-FREE EXTRACT

KLEBSIELLA PNEUMONIAE CELL GROWN ON D-XYLOSE AS DESCRIBED BY JANSEN14 WERE HARVESTED BY CENTRIFUGATION (9750 g, 30 min, 4°C). THE FERMENTATION MEDIUM WAS DISCARDED AND THE CELL PASTE WAS WASHED TWICE IN PHOSPHATE BUFFER (50mM, pH 7.5) AND FROZEN AT −20°C.

FROZEN CELL PASTE WAS MELTED AND SUSPENDED IN AN APPROXIMATELY EQUAL VOLUME OF BUFFER AT 4°C AND THE CELLS WERE Ruptured USING AN ULTRASONIC OSCILLATOR (SONIFER V-185, BRANSON SONIC POWER CO., DANBURY, CT) WITH A MICRO-
tip for 3 min. The sonic extract was then centrifuged (9750g, 20 min, 40°C) the supernatant liquid was decanted and again centrifuged (105,000g, 90 min, 4°C). The precipitate was discarded. The \(1.05 \times 10^6\) g supernatant liquid was decanted and kept at \(-20°C\). At this temperature, dehydrogenase activities were preserved, even after four months. However, acetoin racemase activity was lost. Hence, experiments for measuring acetoin racemase activity used freshly prepared high-speed supernatant liquid.

**Preparation of \(\alpha(\neg)\) Acetoin**

Berl and Bueding\(^6\) reported a procedure to obtain \([\alpha(\neg)]\) acetoin by using an acetone powder of *K. pneumoniae*. This procedure was initially used for this research and later the separation step was modified.

After the pyruvate was converted to acetoin,\(^5\) and the protein had been precipitated, the supernatant liquid was applied to a preparative scale liquid chromatography column (1 × 45 cm) packed with 400-mesh AG50W-X4 (Bio-Rad, Richmond, CA) in the Ca\(^{2+}\) form. The eluent was degassed water at \(85°C\) at a flow rate of \(4\) mL/min. Fractions were collected every \(80\) s. The use of liquid chromatography (LC) to separate the product yielded \(\alpha(\neg)\) acetoin with a specific rotation equivalent to that produced by the distillation procedure of Berl and Bueding.\(^5\)

**Determination of Product Composition**

The stereocomposition of 2,3-BD was determined by using an AG50W-X4 column in the Ca\(^{2+}\) form.\(^9\) The LC detector was set at an attenuation equal to \(1/2 \times \) and \(100-\mu\)L samples were injected. Under these conditions, changes in acetoin or 2,3-BD concentration as low as \(0.02 \text{ mg/mL} \times (2.3 \times 10^{-4}\text{M})\) could be detected. This analysis separates NADH, NAD\(^+\), and other components present in the incubation mixture. The NAD\(^+\) and NADH eluted in three peaks from the column, possibly because of degradation of the NADH and NAD\(^+\) on column. After about 50 injections, resolution between the NAD\(^+\) and nonmeso 2,3-BD peak diminished. The use of a charcoal precolumn to remove the nucleotides and thus protect the LC column was impractical due to peak broadening and subsequent loss of resolution between acetoin and 2,3-BD in the Ca\(^{2+}\) column. Resolution was maintained by packing a new column when necessary.

**RESULTS**

**Fermentation Products and Reduction of Racemic Acetoin**

A fermentation broth of *K. pneumoniae* grown on d-xylene was analyzed to determine the stereocomposition of the 2,3-BD and acetoin produced. Thirty milliliters of fermentation broth were extracted into ether.\(^7\) Thirty milliliters of water were added and the ether was removed in a flash evaporator and dis-
Carded. The remaining aqueous solution was then resolved by preparative scale liquid chromatography (5 x 90-cm column) using the conditions reported for separation of 2,3-BD stereoisomers. Column fractions were analyzed by gas chromatography on Chromosorb 101<sup>b</sup> and by liquid chromatography to verify the presence of a single compound. The nonmeso 2,3-BD identified from its optical rotation as (±) 2,3-BD, ([α]<sub>2</sub>)<sub>21</sub><sup>o</sup> experimental = +14°. [α]<sub>2</sub><sup>o</sup> reported = +13.0°<sup>b</sup>). Both results are consistent with the model proposed by Taylor and Jun<sup>i</sup>. The presence of an acetoin racemase would result in the formation of (±) acetoin and hence explain the lower specific rotation we obtained for the acetoin.

Racemic acetoin (Aldrich Chemical Co., Milwaukee, WI) was reacted with NADH and high-speed supernatant liquid prepared from sonie rupture extracts of K. pneumoniae. Analysis by LC revealed formation of both meso and nonmeso 2,3-BD (Fig. 4). When racemic acetoin was reacted with excess NADH (excess indicates a molar ratio greater than unit), all the acetoin initially present was consumed. Thus, both isomers of acetoin were reduced. These results also are consistent with those predicted by the Taylor and Jun<sup>i</sup> model<sup>e</sup>, since the proposed (±) 2,3-BD dehydrogenase would utilize both

![Fig. 4. An LC chromatogram of a reaction sample after 40 min incubation. The initial incubation mixture contained 1.92mM racemic acetoin, 1.42mM NADH, and high-speed supernatant liquid from K. pneumoniae, all in 50mM phosphate buffer, pH 7.5, at 30°C. Sample size was 100 μL; detector attenuation was ½; chart speed: 12.5 cm/h; m = meso 2,3-BD; α = acetoin; nm = nonmeso 2,3-BD.](image)
acetoin isomers with the reduction of \( \alpha(-) \) acetoin to meso \( 2,3 \)-BD and the reduction of \( \alpha(+) \) acetoin to \( \alpha(+) \) \( 2,3 \)-BD (nonsense BD).

**Separation and Characterization of Two Acetoin Reductases**

Fractionation of high-speed supernatant liquid on DEAE-cellulose yielded two enzyme fractions, E1 and E2. Fraction E1 did not bind to the resin and hence eluted with buffer, but E2 adsorbed on the resin and was recovered by elution with \( 1M \) NaCl. Both fractions oxidized NADH in the presence of racemic acetoin (Fig. 5).

The products of the redox reaction between NADH and racemic acetoin as catalyzed by E1 and E2 were analyzed by I.C. Fraction E1 catalyzed the reduction of racemic acetoin to meso \( 2,3 \)-BD (Fig. 6). Fraction E2 catalyzed reduction of racemic acetoin to nonsense \( 2,3 \)-BD (Fig. 7). Reaction of racemic acetoin, NADH, and mixture of E1 and E2 yielded both meso nonsense \( 2,3 \)-BD. When racemic acetoin was reacted with excess NADH in the presence of E1, only one-half of the acetoin initially present was consumed, as determined by following the change in optical density at 340 nm and calculating a material balance for NADH. This indicates that E1 utilizes only one acetoin isomer.

**Reaction With \( \alpha(-) \) Acetoin**

The \( \alpha(-) \) acetoin \((\alpha)\) was reduced to meso \( 2,3 \)-BD when reacted with NADH in the presence of E1 (Fig. 8). With excess NADH, E1 utilized all the \( \alpha(-) \) acetoin present. Fraction E2 did not catalyze reduction of \( \alpha(-) \) acetoin.

![Fig. 5. DEAE-cellulose (1.5 × 20 cm, Bio-Rad, Richmond, CA) column chromatography of high-speed supernatant liquid of K. pneumoniae. Twenty-seven milligrams of protein was applied to the column, and 4.5-ml fractions were collected. Buffer consisted of 50 mM phosphate, pH 7.5, in 1 mM dithiothreitol. Elution was achieved by a 1M NaCl step in buffer. Enzyme activities shown in E2 are 10×. Enzyme activities are given in IU/ml (1 IU = 1 μmol product/min at 30°C, pH 7.5).](image-url)
Fig. 6. An LC chromatogram of a reaction sample after 25 min incubation. The initial incubation mixture contained 1.92mM racemic acetoin and enzyme fraction E1. Other conditions are as given in Fig. 4.

Fig. 7. An LC chromatogram of a reaction sample after 180 min incubation. The initial incubation mixture contained 1.92mM racemic acetoin, 950uM NADH, and enzyme fraction E1. Other conditions as given in Fig. 4.
To provide corroborative evidence for an acetoin racemase, \( \alpha(-) \) acetoin \((\alpha_{D} = -81^\circ)\) was incubated with sonic extract for 24 h at 30°C. The resulting acetoin was vacuum distilled (30-50 mm Hg) under nitrogen. The distillate had a specific rotation \( \alpha_{D} = -41^\circ \). As a control, buffered sonic extract was replaced by buffer. The mixture was then incubated and isolated under the same conditions. However, a significant decrease in specific rotation was not obtained, \( \alpha_{D} = -80^\circ \). Hence, the change in specific rotation can be attributed to some factor, other than buffer, found in the sonic extract.

In another experiment, \( \alpha(-) \) acetoin was incubated with diatized sonic extract at 30°C for 30 min and a sample was analyzed by LC. In the control, most of the \( \alpha(-) \) acetoin was reduced after 30 min with production of meso 2,3-BD. When \( \alpha(-) \) acetoin was first incubated with the extract, the final products were both meso and nonmeso 2,3-BD. This result indicates that during the 4-h incubation, \( \alpha(-) \) acetoin was converted to its enantiomer, which was then reduced to nonmeso 2,3-BD \((\alpha(+))\) isomer\) by E2.

**DISCUSSION**

Table 1 summarizes the experimental results and Figure 9 shows the proposed model for conversion of acetoin to 2,3-BD by *K. pneumoniae*. The model postulates the presence of an acetoin racemase and two acetoin reductases, one specific for \( \alpha(-) \) acetoin, the other for \( \alpha(+\) acetoin.  

![Diagram](image-url)
Fraction E1 catalyzes reduction of $\alpha(-)$ acetoin to meso 2,3-BD but does not utilize $\alpha(+)$ acetoin. A systematic name for E1 is meso 2,3-butanediol: NAD$^+$ oxidoreductase [$\alpha(-)$ acetoin forming]. Fraction E2 catalyzes the reduction of $\alpha(+)$ acetoin to $\alpha(+) \ 2,3$-BD but does not reduce $\alpha(-)$ acetoin. A systematic name for E2 is $\alpha(+) \ 2,3$-butanediol: NAD$^+$ oxidoreductase [$\alpha(+) \ acetoin$ forming].

The basic difference between the proposed model and that proposed by Taylor and Junö lies in the stereospecificity of acetoin reductases. Neither model allows for conversion of $\alpha(+)$ acetoin to $\alpha(-) \ 2,3$-BD or conversion of $\alpha(-)$ acetoin to $\alpha(+) \ 2,3$-BD. These reactions would require a change in the direction of rotation of the polarized light by the chiral center in acetoin, changes that involve interconversion of two carbon ligand groups (e.g., the hydroxyl and the methyl groups should interchange ligand positions). From the known mechanisms of dehydrogenases, this interconversion would not be anticipated.

\[
\begin{align*}
\text{D}(-) \text{ACETOIN} & \xrightarrow{E_1} \text{L}(+) \text{ACETOIN} \\
\downarrow E_1 & \downarrow E_2 \\
\text{MESO 2,3 BD} & \rightarrow \text{L}(+) \text{2,3 BD}
\end{align*}
\]

Fig. 9. Proposed mechanism for the formation of 2,3-butanediol stereoisomers in K. pneumoniae.
Why should two different acetyl reductases exist? The answer may be related to the different biological functions of each reductase. Halpern and Umbarger\(^4\) reported two different enzyme systems for formation of acetate by *K. pneumoniae*. These exhibit different pH optima and respond differently to thiamine pyrophosphate and Mg\(^{2+}\). One system is identical to that described by Juni\(^5\); the other functions in valine, leucine, and isoleucine biosynthesis.

Application of the proposed model for *K. pneumoniae* to other microorganisms requires the existence of a second \(\alpha(-)\) acetoacet reductase which would reduce \(\alpha(-)\) acetoacet to \(\alpha(-)\) 2,3-BD. This postulate is necessary since \(\alpha(-)\) 2,3-BD is produced in some fermentations (e.g., by *Bacillus subtilis*). This enzyme might also catalyze the reduction of \(\alpha(+)\) acetoacet to meso 2,3-BD (i.e., it would be similar to the \(\alpha(-)\) dehydrogenase proposed by Taylor and Juni\(^5\)) or there might be a fourth enzyme which reduces \(\alpha(+)\) acetoacet to meso 2,3-BD.

The model proposed here predicts that, by reacting racemic acetoacet with excess NADH in the presence of \(\alpha(-)\) acetoacet reductase (E1), the final products, after consumption of \(\alpha(-)\) acetoacet, would be meso 2,3-BD and \(\alpha(+)\) acetoacet. This experiment was performed and indeed gave \(\alpha(+)\) acetoacet.\(^1\)

Experiments carried out by using enzyme fractions, E1 and E2, with 50mM potassium phosphate buffer at pH 5.5, revealed that the enzymes catalyze the same reactions as they did at pH 7.5.

CONCLUSIONS

The model proposed in this work is able to qualitatively explain all the experimental data obtained and the available literature data. It can, therefore, serve as the basis for the enzyme kinetic studies.

References


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