

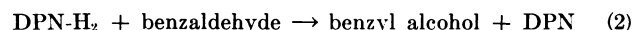
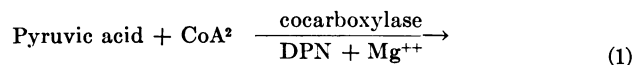
Further Studies on Phenylacetylcarbinol Synthesis by Yeast

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In a recent publication (Smith and Hendlin, 1953) it was shown that the conversion of benzaldehyde to phenylacetylcarbinol by yeasts is accompanied by a concomitant reduction of a portion of the added aldehyde to benzyl alcohol. It was noted further that the two enzyme systems competing for the benzaldehyde were interrelated by their common requirement for coenzyme I (DPN). The oxidized form of DPN serves as the H⁺ acceptor in the dismutation of pyruvic acid and the reduced DPN functions as the H⁺ donor in the reduction of benzaldehyde by alcohol dehydrogenase. Thus:



As pyruvic acid was in excess in the cell-free system, the reduced DPN was indirectly active in the reduction of pyruvic acid to lactic acid, thereby preventing the formation of benzyl alcohol. Furthermore, all the requirements for alcohol dehydrogenase were not met in the cell-free system used for the study of phenylacetylcarbinol synthesis.

The competition of reduced DPN for benzaldehyde during ketol fermentation results in the conversion of a large portion to benzyl alcohol. If the formation of benzyl alcohol were lowered or eliminated, considerably better yields of the ketol could be obtained. This paper reports on the effectiveness of some nicotinic acid analogs in inhibiting the reduction of aldehydes to alcohols both in the fermentation and the cell-free system.

MATERIALS AND METHODS

Acetone powders were prepared from fresh pressed brewers' yeast³ by the method of Hochster and Quastel (1951). Nicotinamide was not added in order to permit the destruction of DPN. The DPN and adenosine triphosphate were obtained from Sigma Chemical Co.⁴ Reduced DPN, used in all experiments with the cell-free

system, was prepared by the hyposulfite method (Le Page, 1949). Nicotinamide was added to the cell-free system to prevent destruction of the DPN by DPNase. The following were present in the cell-free reaction mixture: acetone powder, 5 mg; nicotinamide, m/200; ATP, m/2000; reduced DPN m/2000; phosphate buffer pH 6.5, m/15; aldehyde, 72 μ M. The aldehyde was added after 10 minutes preliminary incubation for temperature equilibration to 28 C. The reaction was allowed to proceed for 30 minutes. Inhibitors were added to the reaction mixture prior to the aldehyde. Controls without any inhibitor were run with each experiment.

Fermentations with whole cells of the fresh pressed brewers' yeast were run in a medium comprised of 86 g refiners' cane molasses per liter adjusted to pH 5.5 with phosphoric acid. To each 250-ml Erlenmeyer flask was added 50 ml medium and 2 g pressed yeast. Following 1 hour prefermentation time at 28 C, 0.2 ml benzaldehyde was added to give a concentration of 4 mg per ml. The fermentation was allowed to proceed for 2½ hours on a rotary shaker at 200 rpm. The broths were then filtered through celite filter aid in a Buchner funnel to remove the yeast cells. The clear broths were retained for analysis of end products. Inhibitors were added prior to the prefermentation period.

Benzaldehyde and benzyl alcohol were determined by the method described previously (Smith and Hendlin, 1953). In the case of benzyl alcohol determinations of the fermentation medium, 20 volumes were steamed distilled from the extract at a neutral pH to obtain a solution of benzyl alcohol free of other ultraviolet absorbable materials. Phenylacetylcarbinol was determined by an iodoform method developed in this laboratory. An aliquot of filtered broth is treated with excess 0.5 N iodine. Excess 10 N NaOH is added and the precipitated iodoform washed with N HCl. The iodoform, dried over calcium chloride in tared centrifuge tubes, is weighed and the amount of ketol calculated. No component of the broths other than phenylacetylcarbinol gives an iodoform reaction.

RESULTS

An interrelationship between the ketol synthesizing system and alcohol dehydrogenase was shown to exist in the cell-free system through a corequirement for DPN. The postulated competition for benzaldehyde

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TABLE 1. Analysis of the fate of benzaldehyde in yeast fermentations

End Product Recovered	Experiment		
	1	2	3
	<i>per cent of benzaldehyde</i>		
Phenylacetylcarbinol.....	18	27	35
Benzyl alcohol.....	65	54	44
Benzoic acid.....	2	2	3
Lost by aeration (by difference).....	15	17	18

during fermentation was proven upon analysis of the fate of the added aldehyde. Low yields of ketol were accompanied by high yields of benzyl alcohol and vice versa. The amount of benzaldehyde lost by aeration and oxidation to benzoic acid remained constant. Recovery experiments are listed in table 1.

It seemed feasible to prevent the reduction of benzaldehyde by alcohol dehydrogenase in three ways: 1) the use of alternate H^+ acceptors; 2) inactivation of the enzyme by $-SH$ inhibitors; and 3) competition with DPN for its enzyme site by nicotinic acid analogs.

Alternate H^+ acceptors. The possibility that a compound, such as an oxidation-reduction dye, might accept H^+ from reduced DPN initially appeared to be the most advantageous method for preventing reduction of benzaldehyde. Among the compounds tested were seven oxidation-reduction dyes (O/R) of different O/R potentials including methylene blue and cysteine, glutathione, acetone, and colloidal sulfur. Only colloidal sulfur was effective, but it proved to be extremely toxic to the over-all fermentation, causing a marked reduction in yields of ketol.

Sulphydryl inhibitors. Since alcohol dehydrogenase is a $-SH$ containing enzyme, it was thought that a specific $-SH$ inhibitor would prevent alcohol formation. However, other enzymes involved in the fermentation of glucose also contain $-SH$ groups. While monoiodoacetic acid at a level of 0.001 M or higher brought about complete inhibition of benzyl alcohol formation in both the cell-free system and the fermentation, it also reduced ketol formation by 90 per cent.

Competition with DPN by nicotinic acid analogs. The use of nicotinic acid analogs to compete with DPN for its enzyme site has the disadvantage of also inhibiting DPN action in reactions necessary for the biosynthesis of phenylacetylcarbinol. For these analogs to be beneficial in decreasing benzyl alcohol formation and increasing the yield of ketol, the effective inhibitor level for alcohol dehydrogenase must be below the concentration inhibitory to ketol synthesis. Because of this fact, the practical usefulness of these compounds seemed doubtful. Nevertheless, several analogs were tested in the cell-free system synthesizing benzyl alcohol and ethanol and in the ketol fermentation. Table 2 lists the

TABLE 2. Inhibition of alcohol formation by nicotinic acid analogs

Nicotinic Acid Analog	Molar Concentration	Per cent Inhibition		
		Cell-free system		Fermentation
		Benzyl alcohol	Ethanol	Benzyl alcohol*
3-Acetyl pyridine.....	0.0005	0	80	22
	0.002	39	100	20
Pyridine-3-sulfonic acid.....	0.0005	14	27	0
	0.002	51	53	14
α -Picolinic acid.....	0.0005	12	27	16
	0.002	13	53	14
Pyrazinamide.....	0.005	100	100	18
Nicotinamide.....	0.05	100	44	21
Isonicotinamide.....	0.05	64	44	15
Isonicotinic acid.....	0.05	37	—	—

* The decrease in benzyl alcohol formation was accompanied by a corresponding increase in phenylacetylcarbinol yields.

inhibitory action of the active compounds. The other analogs tested were nicotinic acid, isonicotinic acid, pyrazinoic acid, pyridazine-5-carboxylic acid, and pyridazine-4-carboxamide. Of the compounds tested, only the amides were capable of antagonizing the action of DPN.

It will be noted that the activity of these compounds is lower in the fermentation phase than in the cell-free system. Higher concentrations of the analogs in the fermentation lower benzyl alcohol yield still further, but they likewise lower the yield of phenylacetylcarbinol.

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SUMMARY

Three approaches were followed in an effort to prevent the reduction of benzaldehyde to benzyl alcohol. The use of compounds that might preferentially accept H^+ from reduced DPN met with failure. Apparently none of these compounds was poised at the proper O/R potential. Inhibition of alcohol dehydrogenase by a $-SH$ inhibitor also reduced the yield of phenylacetylcarbinol. This was suspected since other $-SH$ containing enzymes are concerned in the fermenta-

tation of glucose. Structural analogs of nicotinamide proved to be the most effective means of lowering alcohol yields. These probably acted by competing with DPN for its enzyme site. Only the amides and not the acids of the analogs tested had inhibitory activity. The addition of one of the active analogs to the yeast fermentation decreases alcohol formation and increases ketol yields by 20 per cent. Nevertheless, it was impossible by any of these methods to shunt all of the added aldehyde to the system synthesizing phenylacetylcarbinol.

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Control of Molds During the Enumeration and Isolation of Yeasts from Soil and Plant Material¹

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Most molds (higher fungi) and yeasts are sufficiently acid-tolerant to be easily separated from bacteria by the use of acidified media. However, the enumeration and isolation of yeasts from samples having large populations of molds has always been a problem. Since, as a group, molds grow more rapidly than yeasts and will quickly obscure the surface of an agar plate, it is desirable to have some agent or medium that will inhibit molds or greatly restrict their growth.

Hertz and Levine (1942) compared the effectiveness of sodium propionate and diphenyl in acidified malt extract agar for the separation of pure cultures of yeasts from molds. They noted that sodium propionate was unsatisfactory because concentrations which were sufficiently high to inhibit growth and development of most of the molds also inhibited growth of many of the yeasts. The use of 100 ppm (0.01 per cent) diphenyl in malt extract agar inhibited a large percentage of the

pure cultures of molds studied and restricted the growth of only a few of the yeast species.

In contrast to the results of Hertz and Levine, Mrak and Phaff (1948) reported that a 5 per cent wort agar containing 2,500 ppm (0.25 per cent) sodium propionate was quite effective in the separation of yeasts and molds. More recently, this medium was used by Shihata and Mrak (1952) in the study of the intestinal yeast flora of *Drosophila* and of the yeast flora of plant material and soil.

The present investigation was undertaken with the view of improving existing cultural techniques for separating yeasts from molds in samples from natural sources, such as soil and plant material. Cultural media and techniques were desired that would permit population studies and isolation of the principal yeast species present without interference by mold growth. Since it is not possible to predict the species of yeasts and molds which are likely to be present in a sample, it seemed desirable to use material with unknown flora, as well as pure cultures, in attempts to devise various media which would restrict mold growth.

MATERIALS AND METHODS

Fourteen media were prepared by addition of various agents to dextrose agar (Difco)⁵ as indicated in table 1.

⁵ Difco Laboratories, Detroit, Michigan. Mention of trade products does not imply that they are endorsed or recommended by the Department of Agriculture and the North Carolina Agricultural Experiment Station over similar products not mentioned.

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