Conversion of α-Amino-*n*-Butyric Acid to *n*-Propanol During Alcoholic Fermentation

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Introduction

A rigorous analysis of a California grape brandy fusel oil sample showed that *n*-propanol was present to the extent of 4.1% by weight. The observations of Ehrlich (2) on the α-amino acid origin of other higher aliphatic alcohol components of fusel oil during alcoholic fermentation suggest that α-amino-*n*-butyric acid might be a possible source of *n*-propanol in fusel oil. Although α-amino-*n*-butyric acid has not been identified as a constituent of grapes, it was considered to be of interest to determine whether or not yeast was capable of producing *n*-propanol from synthetic DL-α-amino-*n*-butyric acid.

This paper presents the results of experiments which show that yeast, fermenting glucose under nonproliferating conditions, is incapable of converting α-amino-*n*-butyric acid to *n*-propanol, but can convert a small portion of the amino acid when proliferating in grape juice.

Experimental

Fermentation under Nonproliferating Conditions

Fermentations were carried out under conditions unfavorable for significant yeast multiplication. The basal medium was 0.067 *M* (*M*/15) KH_2PO_4 buffer solution containing 20% glucose, at a pH of 4.5. The yeast used was a strain of *Saccharomyces cerevisiae* var. *ellipsoideus* (Montrachet strain, Enology No. 522), which was employed in an earlier study on higher alcohol formation (3). The yeast was cultivated fermentatively in pure culture in clarified sterile grape juice, nutritionally fortified with Difco yeast extract.

Parallel experimental and control fermentations were set up. Ten grams of DL-α-amino-*n*-butyric acid, C.P., and 40 g. C.P. glucose, each dissolved in *M*/15 KH_2PO_4 solution, were sterilized separately by autoclaving and mixed to give a

TABLE I

Changes in Yeast Cell Count During Fermentations, under Nonproliferating and Proliferating Conditions, with and without Added DL-α-*Amino-n-butyric Acid*

Stage of fermentation	Cell count × 10^6/ml.			
	Nonproliferating fermentations		Proliferating fermentations	
	Amino acid added	Control	Amino acid added	Control
At start	655	713	0.1	0.1
At end	663	853	99.0	82.5
Fractional increase	1.01	1.20	990	825

total volume of 200 ml. The amino acid was omitted from the control. Twenty grams of moist yeast, harvested and washed three times with 3 vol. of phosphate buffer on the centrifuge, was added to each fermentation. Alcoholic fermentation was permitted to proceed with manual agitation of fermentation vessels several times daily. The conversion of sugar to alcohol was found to be complete after 120 hr.

A yeast-cell count using a Levy-Hausser counting chamber, was made at the beginning and end of fermentation. The yeast cell counts are shown in Table I. It is evident that significant yeast cell multiplication did not occur. After completion of fermentation the yeast was removed by centrifuging followed by vacuum filtration through a thin layer of diatomaceous filter aid. The filtrates were submitted to analysis for propanol.

A similar experiment was performed in which the amino acid and glucose were sterilized together by autoclaving. Because of severe browning of the medium, separate sterilization was employed in all other experiments.

Fermentation under Proliferating Conditions

Two 500-ml. portions of Thompson Seedless grape juice (24° Balling, pH 3.6), clarified by heating and filtration, were placed in 2-l. Erlenmeyer flasks plugged with cotton. The juice was fractionally sterilized by heating in flowing steam for 30 min. on each of three successive days. To one portion of sterile juice (experimental) was added 50 ml. of distilled water solution containing 10 g. of DL-α-amino-*n*-butyric acid. To the other portion (control), 50 ml. of sterile distilled water was added. One milliliter of actively fermenting pure yeast culture, propagated in sterile grape juice, was added to each flask. Fermentation was permitted to proceed at 28°C. with periodic agitation daily for 144 hr., at which time evolution of carbon dioxide had ceased. Yeast cell multiplication, which is usually complete in 48 hr. under such conditions, occurred as indicated in Table I. After fermentation the yeast was removed from the fermented medium as described above, and the filtrates were submitted to analysis.

Analysis for n-Propanol

The fermentation filtrate, with a trace of Dow Corning Antifoam A added, was distilled in an all-glass distillation apparatus until the temperature rose to 100° and remained there for an additional 15 min. The aqueous distillate was saturated with sodium chloride and continuously extracted with ethyl ether for 2 days with three changes of ether during the extraction period. The ether extract was dried over anhydrous magnesium sulfate and, after removal of the drying agent, 20 ml. of absolute ethanol (>99.95%) was added to remove the last traces of moisture as the ethanol–water azeotrope (1) during the subsequent distillation. Ten milliliters of synthetic isoamyl alcohol was added to act as a chaser for any *n*-propanol present, and the mixture was then fractionally distilled through a micro Podbielniak column until the head temperature rose to the boiling point of isoamyl alcohol. Since no distillation plateau which would correspond to the boiling point of *n*-propanol was observed in any of these fractionations the material boiling over the range 78–132° was collected in each instance and analyzed for the presence of *n*-propanol. A small portion of this fraction was converted into the 3,5-dinitrobenzoate derivative (1), and the entire derivative sample was analyzed by chromatography on a silicic acid column according to the method of White and Dryden (4).

Qualitative Chromatography of Added Amino Acid

Paper strip chromatography was employed to determine whether or not gross changes in the amounts of added α-amino-*n*-butyric acid had occurred during fermentation. The residues from the first distillation of the fermented media were adjusted to the original volume. Ten-microliter spots were run by the descending technique on Whatman No. 1 paper with phenol saturated with water as developing solvent. A ninhydrin spray was used to develop color on the paper. Experimental and control samples were compared with solutions of DL-α-amino-*n*-butyric acid of known concentration.

RESULTS

Nonproliferating Fermentation

From the fractionation of the fermentation with added amino acid cited in Table I, 710 mg. of material boiling over a range 78–132°C. was collected. Chromatography of the 3,5-dinitrobenzoate derivative of this material gave two sharp, well-separated zones, material from the upper of which melted at 90–92°C. (mixed m.p. with ethyl 3,5-dinitrobenzoate: 91–92.5°C.), and from the lower at 61–62°C. (mixed m.p. with isoamyl 3,5-dinitrobenzoate: 60.5–61.5°C.). No intermediate zone which would indicate the presence of *n*-propyl 3,5-dinitrobenzoate could be detected on the column.

Analysis of the control fermentation (without added amino acid) gave no evidence for the presence of *n*-propanol. The same results were ob-

tained in the experiment in which the amino acid and sugar were sterilized in solution together.

Proliferating Fermentation

Fractionation of the material from the fermentation with added amino acid gave 570 mg. of a fraction with a boiling range of 78–132°C. Chromatography of the 3,5-dinitrobenzoate derivative of this fraction gave three clearly separated zones which were eluted through the column, and the weight of material from each zone was determined. The material from the upper and lower zones were the ethyl and isoamyl 3,5-dinitrobenzoate derivatives, respectively, while the material from the middle zone was *n*-propyl 3,5-dinitrobenzoate (m.p. 70–72°C.; mixed with *n*-propyl 3,5-dinitrobenzoate: m.p. 71–72.5°C.).

On the assumption that the conversion of the alcohols to the derivatives was completed to about the same degree for each of the primary alcohols involved, and that the weights of material from the bands on the column gave the relative amounts of each derivative (1), the break fraction was calculated to contain 81 mg. of *n*-propanol. The theoretical amount of *n*-propanol that could result from complete conversion of one isomer of the added amount of DL-α-amino-*n*-butyric acid was 2.92 g. Thus the yield of *n*-propanol based on the amount of added amino acid was 2.8 %, and on the weight of ethanol produced, 0.16 %.

Amino Acid Residual in Fermentation Medium

For each experimental fermentation (added amino acid), the size and color intensity of the spot produced on paper chromatograms was not visually distinguishable from the spot produced by the known equivalent solution of amino acid. The result indicated no gross changes in amounts of the amino acid added to the fermentations. Control fermentation residues showed no spots corresponding to the position of the DL-α-amino-*n*-butyric acid spot.

Discussion

Ehrlich (2) has reported that yeast (presumably a variety of *Saccharomyces cerevisiae*) was capable of transforming leucine to isoamyl alcohol under nonproliferating conditions, with yields of nearly 100 % on the basis of conversion of one-half of the DL-leucine added. This efficiency in the utilization of leucine is in sharp contrast with the efficiency of utilization of α-amino-*n*-butyric acid, which we find to be less than 0.03 %

under similar conditions. It is suggested that the difference in utilization of the two amino acids is caused by the lack of a specific enzyme system for utilization of α-amino-*n*-butyric acid. The experiments which were run under conditions permitting yeast cell multiplication resulted in the formation of at least 100 times as much *n*-propanol as was formed under conditions permitting fermentation but practically preventing multiplication. These results might be taken to support Ehrlich's proposal that the formation of the higher alcohol components of fusel oil is essentially a consequence of the protein-building activity of the *Saccharomyces* yeast cell (2), or that an adaptive enzyme, which can only be formed under conditions permitting growth, is required for the decomposition of α-amino-*n*-butyric acid. If the latter explanation is correct, it should be possible to develop the adaptive enzyme system to such an extent that larger amounts of α-amino-*n*-butyric acid could be converted to *n*-propanol by the yeast cells. This point is under investigation.

In addition to the conclusions concerning the utilization of α-amino-*n*-butyric acid by yeast cells, the experimental results permit certain other interesting deductions to be made. Since there was no evidence of the formation of *n*-propanol in the control fermentations in which grape juice without amino acid was fermented, and no spot corresponding to α-amino-*n*-butyric acid was found when the control grape juice fermentation was chromatographed on paper, it is apparent that there is little or no α-amino-*n*-butyric acid in the juice of Thompson Seedless grapes. The absence of *n*-propanol in this control fermentation would also indicate that neither free *n*-propanol nor any compound readily hydrolyzable during fermentation or distillation to give *n*-propanol could have been present in the grapes employed. The presence of *n*-propanol in appreciable quantities in the commercial fusel oils may well be the result of bacterial action on substances other than α-amino-*n*-butyric acid which are present in grapes.

Summary

In sterile grape juice medium a strain of *Saccharomyces cerevisiae* var. *ellipsoideus*, under proliferating conditions, converted a small portion of added α-amino-*n*-butyric acid to *n*-propanol. The yield was 2.8 % on the basis of utilization of one isomer of the synthetic α-amino-*n*-butyric acid added, or 0.16 % on the basis of the ethanol produced. Without added amino acid, no detectable *n*-propanol was formed. In sterile glucose–phosphate buffer solution containing added amino acid, the metabolic

activity of heavy inoculations of the same yeast did not produce a detectable amount of *n*-propanol.

References

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