The Formation of Higher Alcohols in the Fermentation of Amino Acids by Yeast.

The Determination of Alcohols with Gas Chromatography.

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We have found that some straight-chained α -amino acids are converted by yeast to the alcohols with correspondingly longer carbon chains in the alcoholic fermentation contrary to F. Ehrlich's scheme, i.e., isobutyl alcohol from alanine and active amyl alcohol from α -amino-*n*-butyric acid or threonine.

In this report, we confirmed this fact in the alcoholic fermentation of many aliphatic amino acids by 2 yeast strains using gas chromatography. Moreover, *n*-propyl alcohol was proved to come from α -amino-*n*-butyric acid or threonine. Small quantities of *n*-propyl, isobutyl, active amyl and isoamyl alcohols were found in all the fermented solutions. There was some difference in the composition of higher alcohols of the alcoholic solutions fermented by different yeasts.

INTRODUCTION

At the beginning of this century F. Ehrlich¹⁾ showed that in the alcoholic fermentation of sugar by yeast the production of isoamyl alcohol was stimulated by the addition of leucine to the medium and was suppressed by the addition of inorganic sources of nitrogen. He proposed that higher alcohols were formed from amino acids by a process of deamination. Neubauer and Fromherz²⁾ studied the mechanism and proposed a scheme of reactions through which the amino acid was oxidatively deaminated to form a keto acid which was subsequently decarboxylated and reduced, yielding a higher alcohol with a carbon chain length reduced by one compared with that of amino acid. Afterwards R. Thorne³⁾ ascertained this scheme in many amino acids. Re-

cently Sentheshanmuganathan⁴⁾ demonstrated the essential validity of the scheme. Using cell-free extracts he showed that tyrosine underwent transamination with α -ketoglutaric acid to *p*-hydroxyphenyl pyruvic acid then this acid was decarboxylated and reduced to yield tyrosol.

We⁵⁾ also studied this problem long ago and showed that some straight-chained aliphatic amino acids did not obey this scheme, but gave rise to higher alcohols with correspondingly longer carbon chains, i.e., isobutyl alcohol from alanine and active amyl alcohol from α -amino-*n*-butyric acid or threonine. But the yeast used in these experiments was different from those used by previous workers. They used brewer's or baker's yeast and we used saké yeast, though they all belong to

1) F. Ehrlich, *Ber.*, **40**, 1027 (1907).

2) O. Neubauer and K. Fromherz, *Z. physico. Chem.*, **70**, 326 (1910).

3) R. Thorne, *J. Inst. Brewing*, **43**, 288 (1937).

4) Sentheshanmuganathan and S. Elsdon, *Biochem. J.*, **69**, 210 (1958). Sentheshanmuganathan, *ibid*, **74**, 568 (1960).

5) M. Yamada, *This Journal* **8**, 95 (1932), **11**, 24 (1935); M. Yamada, K. Yoshizawa and S. Sasaki, *ibid*, **22**, 314 (1958); M. Yamada and K. Yoshizawa, *ibid*, **24**, 266 (1960).

same species, *Saccharomyces cerevisiae*. More-over analytical methods available at that time were not sufficient to gain the clear separation and determination of each alcohol. Now the development of analytical techniques, especially the application of gas chromatography on this area, makes it possible to obtain the result more successfully. So in this report we have repeated the previous experiments using gas chromatography. Two strains of yeast, saké yeast and brewer's yeast, were used for the purpose of supplementing the data of previous experiments.

EXPERIMENTAL

Yeast: In regard to contradictory results obtained in the alcoholic fermentation of alanine by R. Thorne⁶⁾

and us⁵⁾ Thorne suggested in his letter that the different yeast strains used in the experiments might possess different pathways through which the amino acid is converted. So we used 2 strains of yeast, saké yeast Kyôkai No. 7 and brewer's yeast used in brewing factories in Japan. No addition of vitamin is necessary for the growth of the former strain, but the latter requires Ca pantothenate.

Materials: The majority of amino acids were given by Ajinomoto Co., Ltd. Alfa-amino-isobutyric acid was synthesized⁷⁾. Isoleucine used contained 55% of isoleucine and 45% of leucine. Alcohols used as the standard materials were distilled with a fractional distiller with 70 theoretical plates. Active amyl alcohol was synthesized by the method described for the synthesis of cyclohexyl carbinol⁷⁾ using 2-methyl propyl bromide⁸⁾ instead of cyclohexyl chloride.

Methods: To each 1 l of medium, which contained 100 g of sucrose, a nitrogen source with the amount of

TABLE I. THE COMPOSITION OF THE MEDIA BEFORE AND AFTER FERMENTATION

Nitrogen Source	Yeast	Before Fermentation					After Fermentation					
		N-Source g/l	Acidity	Formol -N	pH	Yeast Innoc.	Yeast Gained	Alc. %	Acidity	Formol -N	pH	Fusel Oil ml/l
Ammonium Sulphate	{S*	3.0	0.35		5.2	0.4***	5.0****	5.0	3.8		2.4	0.12
	{B						5.5	4.2	2.85		2.8	0.10
Yeast-Acid Hydrolysate	{S	1.5	0.20	1.1**	5.8	4.5	10.0	5.5	3.0	0.15	3.6	0.16
	{B						7.4	6.0	4.0	0.2	3.0	0.24
Glycine	{S	1.7	0.15	2.4		3.7	4.2	5.0	3.4	1.5	3.2	2.04
	{B						6.35	4.8	3.3	1.75	2.6	
L-Alanine	{S	1.15	0.35	1.1	5.2	4.7	5.0	5.0	2.4	0.15	3.2	0.96
	{B							5.4	1.75	0.15	3.2	0.60
DL- α -Amino-n-but. Acid	{S	2.3	0.25	1.9	5.0	4.7	5.5	5.0	3.7	1.0	3.2	1.74
	{B						12.5	5.1	2.9	0.72	2.8	1.20
DL-Threonine	{S	3.1	0.22	1.85	5.2	4.1	10.0	4.5	2.5	1.0	3.2	0.96
	{B						7.75	3.3	1.5	0.7	3.0	0.60
L-Valine	{S	1.4	0.30	1.1	5.0	4.7	4.3	5.0	2.0	0.15	3.8	2.16
	{B						8.3	5.2	2.95	0.10	2.8	
DL-n-Valine	{S	2.6	0.15	1.8	5.0	3.5	10.8	4.5	2.8	0.8	2.8	0.54
	{B						8.1	5.2	2.0	0.75	3.0	0.28
L-Leucine	{S	1.5	0.25	1.05	5.0	4.7	8.6	5.5	2.4	0.1	3.6	1.86
	{B						11.6	5.0	1.8	0.15	3.2	1.32
L-Isoleucine	{S						11.1	5.5	2.3	0.1	3.6	1.92
	{B						7.2	4.8	2.3	0.15	3.0	0.60
L-Asp. Acid	{S	1.5	0.25	1.05	5.0	4.1	10.0	5.5	1.7	0.1	4.2	0.66
	{B						6.85	5.2	1.7	0.2	3.2	0.46
L-Glu. Acid	{S	3.5	0.15	1.15		3.7	10.8	5.5	2.8	0.15	4.4	0.72
	{B						19.5	5.5	2.65	0.35	3.0	0.30

* S; Sake Yeast, B; Brewery Yeast.

** Ml. of 1/10 N. NaOH used for titration of 10 ml of sample.

*** The number of yeast inoculated per ml, multiplied by 10⁸.

**** The number of harvested yeast per ml, multiplied by 10⁷.

6) H. Gilman, *Org. Syn.*, Col. Vol. 2, 29 (1955).

7) H. Gilman, *ibid.*, Col. Vol. 1, 188 (1956).

8) H. Gilman, *ibid.*, Col. Vol. 2, 247 (1955).

assimilable nitrogen equal to a half of that of 3 g of *dl*-aspartic acid, 20 ml of Hayduck mineral solution, 0.2 mg of Ca pantothenate, 2 γ of biotin, 5 mg of inositol and water, the yeast suspension which contained about 4,000,000 of precultured yeast was added. The fermentation was carried out in 25°C for 10~14 days with occasional shaking. α -Amino-isobutyric acid was not fermented by either yeast as we reported previously⁹⁾, but glycine was fermented although the fermentation period was rather long. After fermentation the yeast cells were removed and the amounts of alcohol, amino acid and fusel oil were determined. The acidity and the pH of the fermented solution were also examined. Table I shows the constituents of the media before and after fermentation. Fusel oil was determined by the colorimetric procedure of Sada⁹⁾, Teramoto¹⁰⁾, Yoshizawa and Iwatsuki¹¹⁾ which is based on the color developed by the reaction of higher alcohols upon heating at 100°C for 3 min. with 0.5% vanilline in concentrated sulfuric acid (Komarowsky reaction).

The fermented solution was distilled to concentrate alcohol. When the concentration of alcohol in the distillate was over 80%, the distillate was fractionally distilled with a 50 cm long Widmar column. After the temperature at the top of column reached 79°C, collection of the higher alcohol fraction was begun and continued until the temperature reached 100°C. During the distillation the loss of higher alcohol was examined by the colorimetric method of reacting the distillate or residue with a vanilline-sulfuric acid reagent⁹⁻¹¹⁾.

The distillate thus obtained was used directly for gas chromatographic analysis. The apparatus was Kotaki Super Fractioner Type GU-21. Using a 2.5 m dioctyl phthalate column, *n*-propyl, isobutyl, *n*-butyl and amyl alcohols were fairly well separated as shown in several examples in Fig. 1. The separation of isoamyl and active amyl alcohol was not accomplished with this column and therefore 8 m glycerol column¹²⁾ was used to separate these alcohols from each other. For this procedure the sample was dried overnight with anhydrous sodium sulphate. The good separation was obtained as shown in Fig. 2. Aldehydes and esters contained in the higher alcohol fraction were

so little that they did not interfere with the separation of alcohols in gas chromatography. The percentage of each component was estimated by the area calculated by multiplying the height of peak with the width measured at one half the peak height after correction with a calibration curve obtained with pure alcohols previously. The identification was established by recording the retention times of components of known mixtures and was further confirmed by addition of individual pure alcohols to the unknown mixtures.

RESULTS AND DISCUSSION

Table II summarizes the results obtained with gas chromatography. A large quantity of fusel oil was produced in the medium of glycine, valine, leucine, α -amino-*n*-butyric acid, threonine, isoleucine or alanine. In the media of ammonium sulphate and yeast acid-hydrolysate a small quantity of higher alcohols was produced. Ordinarily saké yeast produced a larger quantity of higher alcohols than brewer's yeast. Whatever nitrogen source was used in the fermentation medium *n*-propyl, isobutyl, active amyl and isoamyl alcohol were always found in the higher alcohol fractions, in which usually the largest fraction, isoamyl alcohol, occupied 35 to 50% of the alcohol content. Isobutyl alcohol occupied 20 to 40% and active amyl alcohol occupied 10 to 20%. Normal propyl alcohol was usually the smallest fraction. But these percentages changed in the higher alcohol fractions formed from some amino acids. The higher alcohol fraction produced from leucine by either yeast consisted mainly of isoamyl alcohol. Isobutyl and active amyl alcohol occupied only 5% of the total alcohol content. The higher alcohol fraction formed from valine fermentation by both yeast contained isobutyl alcohol up to 87% and small quantities of *n*-propyl, active amyl and isoamyl alcohol. In the fermentation of *n*-valine by both yeasts the higher alcohol formed was mostly *n*-butyl alcohol, but the percentage of the *n*-butyl alcohol in the higher alcohol fraction was different between

9) R. Sada, *Report Brew. Exp. Station*, **44**, 1 (1912).

10) S. Teramoto, *J. Ferm. Tech.*, **17**, 323 (1939).

11) K. Yoshizawa and M. Iwatsuki, *J. Soc. Brewing, Japan*, **54**, 691 (1959).

12) J. Ingraham and J. Guymon, *Archives Biochem. Biophys.*, **88**, 157 (1960).

FIG. 1. Separation of Alcohols by Gas Chromatography Using a 2.5 m Dioctyl Phthalate Column; Helium flow 82 ml/min.; 105°C.

Vertical: relative recorder response.

Horizontal: retention time-minutes.

Et: ethyl alcohol; H₂O: water; *n*-Pr: *n*-propyl alcohol; *i*-Bu: isobutyl alcohol; *n*-Bu: *n*-butyl alcohol; *a*-Am: active alcohol; *i*-Am: isoamyl alcohol.

FIG. 2. Separation Active Amyl and Isoamyl Alcohols by Gas Chromatography Using a 8 m Glycerol Column; Helium flow 62 ml/min.; 75°C

Vertical: relative recorder response.

Horizontal: retention time-minutes.

n-Pr: n-propyl alcohol; a-Am: active amyl alcohol; i-Am: isoamyl alcohol.

TABLE II. THE COMPOSITION OF FUSEL OIL

Nitrogen Source	Yeast	Fusel Oil ml/l	Percentage in Fusel Oil					Ml. per l. of Medium					Note
			<i>n</i> -Pr	<i>i</i> -Bu	<i>n</i> -Bu	<i>ac</i> -Am	<i>i</i> -Am	<i>n</i> -Pr	<i>i</i> -Bu	<i>n</i> -Bu	<i>ac</i> -Am	<i>i</i> -Am	
Ammonium Sulphate	{S*	0.12	15.7	30.0		35.8	18.5	0.02	0.04		0.03	0.05	Unknown Peak (Rt.** 7.3 m.)
	{B	0.10	13.3	22.3		19.4	45.0	0.01	0.02		0.02	0.05	
Yeast-Acid Hydrolysate	{S	0.16	2.6	33.8		16.0	47.6	0.004	0.05		0.03	0.08	
	{B	0.24	6.1	24.5		17.4	52.0	0.02	0.06		0.04	0.14	
Glycine	{S	2.04	Tr.***	47.8		13.2	39.0	Tr.	0.98		0.26	0.80	
	{B		Tr.	46.5		11.8	41.7						
L-Alanine	{S	0.96	5.5	55.5		13.5	25.5	0.06	0.56		0.14	0.26	Unknown Peak (Rt. 7.3 m.)
	{B	0.60	3.2	39.9		20.2	36.7	0.02	0.24		0.13	0.23	
DL- α -Amino- <i>n</i> -but. Acid	{S	1.74	16.9	8.5		63.9	10.7	0.36	0.18		1.34	0.22	Unknown Peak (Rt. 7.3 m.)
	{B	1.20	66.1	10.8		20.1	3.0	2.34	0.38		0.71	0.11	
DL-Threonine	{S	0.96	12.4	8.6	1.3	66.3	11.4	0.14	0.10	0.01	0.73	0.13	
	{B	0.60	26.5	4.9	2.1	52.2	14.3	0.22	0.04	0.02	0.44	0.12	
L-Valine	{S	2.16	5.6	86.9		2.0	5.5	0.13	1.98		0.05	0.13	
	{B		8.1	87.2		1.4	3.3						
DL- <i>n</i> -Valine	{S	0.54	Tr.	10.3	75.4		13.6	Tr.	0.22	1.70			
	{B	0.28	Tr.	3.5	87.7		8.8	Tr.	0.08	2.00			
L-Leucine	{S	1.86	Tr.	5.3		Tr.	94.7	Tr.	0.10		Tr.	1.76	
	{B	1.32	Tr.	2.0		3.5	94.5	Tr.	0.02		0.06	1.24	
L-Iso leucine	{S	1.92	Tr.	5.2		46.4	48.4	Tr.	0.10		0.89	0.93	
	{B	0.60	Tr.	2.5		50.9	46.6	Tr.	0.12		0.31	0.27	
L-Asp. Acid	{S	0.66	2.4	38.8		14.4	44.4	0.02	0.26		0.01	0.30	
	{B	0.46	6.6	31.9		14.9	46.6	0.03	0.16		0.07	0.23	
L-Glu. Acid	{S	0.72	7.8	31.4		12.2	48.6	0.06	0.24		0.10	0.38	Unknown Peak (Rt. 9.5 m.)
	{B	0.30	9.9	30.1		11.6	48.4	0.03	0.10		0.04	0.16	

* S; Sake Yeast, B; Brewer's Yeast.

** Rt; Retention Time. m.; Minute.

*** Tr; Trace.

2 yeasts. In the fermentation of isoleucine the formed higher alcohol consisted of nearly equal quantity of isoamyl and active amyl alcohol. As isoleucine used for this experiment consisted of 55% isoleucine and 45% leucine, it is certain that in the fermentation of these amino acids higher alcohols are formed mainly through the pathway proposed by Ehrlich.

But in alanine, α -amino-*n*-butyric acid and threonine, the formation of higher alcohol does not seem to obey Ehrlich's scheme. The largest constituent of the higher alcohol formed from alanine was isobutyl alcohol, i.e., 55.5% in saké yeast and 39.9% in brewer's yeast. There were other higher alcohol fractions of which the largest component was isoamyl alcohol. In the higher alcohol formed from α -amino-*n*-butyric acid by saké yeast a large quantity of active amyl alcohol (62.9%

of higher alcohol) was formed. In the case of brewer's yeast 66.1% of the higher alcohol fraction was *n*-propyl alcohol. The quantity of active amyl alcohol was much higher than that in higher alcohol fractions from other amino acids except threonine and isoleucine. More than half of the higher alcohol formed from the fermentation of threonine by either yeast was active amyl alcohol, although a fairly large quantity of *n*-propyl alcohol was present. A small quantity of *n*-butyl alcohol was also found.

According to Ehrlich's scheme ethyl alcohol is expected to be formed from alanine and *n*-propyl alcohol from α -amino-*n*-butyric acid or threonine. But the results mentioned above show that side-chained higher alcohols with correspondingly longer carbon chains than the original amino acids are formed in the fermen-

tation of these amino acids as we reported previously⁵). But as shown in Table II a considerable quantity of higher alcohol was also formed through the pathway proposed by Ehrlich and others in the fermentation. It is interesting that saké yeast produced a larger amount of the higher alcohols with correspondingly longer carbon chains from amino acids than brewer's yeast did.

In the fermentation of glycine the largest constituent of higher alcohol was isobutyl alcohol as in the case of alanine. This problem will be further investigated. Some unknown peaks were found on gas chromatograms of the higher alcohol fractions of the ammonium sulphate, α -amino-*n*-butyric acid and glutamic acid media after fermentation by saké yeast. They seem to be due to the same compound except that of the glutamic acid medium, but were present in too small a quantity to be determined.

SUMMARY

1. Higher alcohols formed in the fermentation of media containing sucrose and various nitrogen sources by 2 different yeast strains were analysed and determined with gas chro-

matography.

2. Glycine was fermented yielding a large quantity of higher alcohols, but α -amino-isobutyric acid was not fermented.

3. Higher alcohol fractions from all the media after fermentation contained *n*-propyl, isobutyl, active amyl and isoamyl alcohols. Usually the largest component was isoamyl alcohol and smallest was *n*-propyl alcohol.

4. The formation of higher alcohols from valine, *n*-valine, leucine and isoleucine followed largely Ehrlich's scheme.

5. The higher alcohols with correspondingly longer carbon chains were produced from the fermentation of several straight-chained aliphatic amino acids, isobutyl alcohol formed from alanine, active amyl alcohol formed from α -amino-*n*-butyric acid or threonine.

6. Normal propyl alcohol was shown to be formed also from α -amino-*n*-butyric acid or threonine.

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