STUDIES ON THE FERMENTATION PRODUCTS BY MOULD FUNGI. IV.

ASPERGILLUS GLAUCUS. PART I.

By

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I. Condition of the Cultivation.

Aspergillus glaucus was cultured at 30°C during 10—180 days in the following medium.

C-source : glucose or saccharose, 10%.

N-Source : Na-nitrate, -nitrite, Am.-nitrate, -sulphate, peptone, urea, asparagine, aspartic acid, glutamic acid, glycocoll, leucine, tyrosine, alanine, etc. 0.08—2.0%

Mineral matter . K-monophosphate & -diphosphate, each 0.015%.

Mg-sulphate & Ca-chloride, each 0.010%.

Fe-chloride & Na-chloride, each trace.

Ca-carbonate, if necessary.

II. Isolation of the Fermentation Products.

(a) The fermented medium is filtered, added with sulphuric acid and distilled with steam to isolate volatile substances from nonvolatile substances. The residue is evaporated to a small quantity and extracted with ether. After removal of ether, the crystal is dried on the tile and its melting point was examined (the m. p. of mixture with pure substance is also observed). When the crystals are the mixtures of 2 or more acids, they are isolated by adding Ca-acetate and Ca-hydroxide (see Bull. Agri. Chem. Soc. Japan., **2**, 94, 1926).

(b) The fermented medium is evaporated, neutralized with ammonia and added with Pb-acetate. Ca-gluconate and -lactate remain in the filtrate. The filtered precipitate is treated with dil. acetic acid at 70°. Pb-malate goes into solution. The in acetic acid insoluble part of Pb-ppt. is treated with SH₂. After evaporation of this filtrate Ca-oxalate is precipitated by addition of acetic acid and Ca-chloride. After 24 hours the ppt. is filtered and from the filtrate tartaric acid is precipitated as wein stone adding K-acetate and 2 volumes of alcohol. After addition of ferric chloride and removal of Fe-

succinate, the filtrate is acidified, extracted with ether and then from evaporated solution citric acid is precipitated as Ba-citrate by adding Ba-acetate and 3 volumes of alcohol.

III. Identification of the Fermented Products.

1. Ethyl alcohol and volatile acid.

Volatile acid is not produced, but nearly trace of ethyl alcohol is produced, for the distillate of the steam distillation shows neutral reaction with litmus paper and gives jodeform reaction after 24 hours but does not change the colour of Schiff's reagent.

2. Fumaric acid.

M. p. : 282—3° (sealed capillary, recrystallized from hot water).

Ag-salt.	Subst.	0.5856 g.	AgCl	0.5020 g.
	$C_4H_2O_4Ag_2$	Cal.	Ag	65.40%.
		Fou.	Ag	64.46%.

3. Succinic acid.

M. p. : 183—4° (recrystallized from hot water).

Ag-salt.	Subst.	0.1024 g.	AgCl	0.0878 g.
	$C_4H_4O_4Ag_2$	Cal.	Ag	65.06%.
		Fou.	Ag	64.54%.

4. Citric acid.

M. p. : 153—4° (recrystallized from hot water and dried in the vacuum decicator over sulphuric acid).

Ag-salt.	Subst.	0.5996 g.	AgCl	0.5010 g.
	$C_6H_5O_7Ag_3$	Cal.	Ag	63.12%.
		Fou.	Ag	62.89%.

5. Oxalic acid.

Hydrous crystal melts at 100° and the m. p. of the mixture with pure hydrous oxalic acid shows no depression. The colour reaction by ferric chloride is positive.

6. Tartaric acid.

Anhydrous crystal melts at 168° and the m. p. of mixture with pure tartaric acid shows no depression. It gives the reaction of resorcin- H_2SO_4 by heating.

7. Malic acid.

The melting points of the crystal and of the mixture with pure malic acid are both 100°. It gives diazo-reaction without heating.

8. 2-oxymethyl furane-5-carbonic acid.

By the method of isolation (a), the crystal which melts at 150—8° is obtained in some cultures. Recrystallizing from boiling water it crystallizes in long plate or

prism and melts at $163-4^{\circ}$ (incorr.). It contains no nitrogen.

The elementary analysis is as follows :

1. Subst.	0.1402 g.	CO ₂	0.2604 g.	H ₂ O	0.0534 g.
2. "	0.1398 g.	"	0.2604 g.	"	0.0564 g.
1.	C% = 50.66,	H%	= 4.23.		
2.	" 50.80,	"	4.48.		
aver.	" 50.73,	"	4.36,	O%	= 44.91.

Thus, C : H : O = 1.5 : 1.5 : 1. $\therefore$ Empirical formula = $C_3H_3O_2$.

Determination of the molecular weight is as follows :

1. 0.0584g Subst. requires to neutralise 3.90c.c. of ca. N/10 KOH, i.e. 3.8965 c.c. of N/10 KOH (indicator : phenolphthalein). $\therefore$ Mol. weight, = 149.
2. Subst. 0.0128g. Camphor 0.1274g. $\Delta T = 28^{\circ}$ $\therefore$ M. w. = 144.
3. " 0.0158g. " 0.1512g. " = 27° " 144.

From the above results the molecular formula for this substance is given as $C_6H_6O_4$.

The existence of "furane nucleus" is proved by the colour reaction of isatin- H_2SO_4 (violet colour).

The existence of double bond is also proved by absorption of bromine and decolorisation of K-permanganate.

Moreover, the melting point of the mixture with pure 2-oxymethyl furane-5-carbonic acid (m. p. $163-4^{\circ}$) which I synthesized (Fenton & Robinson : Jour. Chem. Soc., **95**, 1339, 1909 ; Fenton & Gostling : Jour. Chem. Soc., **75**, 429, 1899) shows no depression. Therefore it is an established fact that the crystal melting at $163-4^{\circ}$ is 2-oxymethyl furane-5-carbonic acid.

9. An unknown substance.

It is a colourless crystal melting at $238-9^{\circ}$ (incorr.) which contains no nitrogen. It is soluble in ether, alcohol but insoluble in water or boiling water. It is easily purified by dissolving in boiling water and then in alkali and precipitating by acid. I promise here that there will be a chance to write its constitution another day.

IV. Estimation of the Fermented Product.

The medium used is as follows :

1. Am.-sulphate	0.08%.	3. Peptone	0.08%.	5. Na-nitrate	0.08%.
2. "	2.00%.	4. "	2.00%.	6. "	2.00%.

The amounts of acids which were produced was titrated with ca. N/10 NaOH, using phenolphthalein as indicator and showed in the following table as c.c. of ca. N/10 NaOH in 100 c.c. of medium.

The amount of glucose which remained in the medium was estimated by Bertrand's method and showed as g. in 100 c.c. of medium in the following table.

	days.	4	6	8	10	12	14	17	19	23	25	27	29
1	{acid	2.5	3.0	3.5	4.5	5.5	4.5	5.5	7.0	7.0	7.6	8.0	10.5
	{glucose	6.78	6.59	6.45	6.35	6.35	6.29	6.29	6.18	5.85	5.85	5.85	4.23
2	{acid	6.5	7.0	9.0	9.5	9.5	10.0	10.0	14.0	14.0	10.5	10.5	10.5
	{glucose	6.78	6.59	6.59	5.85	5.65	5.52	5.40	5.28	5.28	5.28	5.28	5.28
3	{acid	0	0	2.0	4.0	7.5	15.0	17.5	18.0	22.0	25.0	25.0	30.5
	{glucose	6.78	6.52	6.42	6.12	5.83	5.52	5.52	5.28	4.95	4.93	4.85	5.23
4	{acid	0	0	15.0	39.5	28.0	28.0	14.5	0	0	—	—	—
	{glucose	5.40	4.93	2.62	1.82	0.66	0.64	0.53	0.48	0.35	—	—	—
5	{acid	0.25	0.5	0.5	0	0	2.0	48.0	38.0	23.0	—	—	—
	{glucose	7.64	7.45	7.19	7.15	6.59	6.07	5.82	5.45	5.09	—	—	—
6	{acid	0	0	0	0	0	3.2	3.8	8.0	14.4	—	—	—
	{glucose	6.95	6.55	5.60	5.59	5.72	5.72	2.95	2.16	1.02	—	—	—

V. Pigment.

About 10 years ago, Mr. Klöcker found that this fungus produced the pigment similar to fluorescein (Centbl. f. Bakt., 2 Abt. **46**, 1918). I wished to isolate as a crystal this pigment but could not.

First of all, I tried to discover the best condition to produce this pigment and found among the source of nitrogen peptone or Na-nitrate is the best. The effects of metallic salts were also examined and it was found that Sn-chloride, Al-sulphate, Pb-nitrate, Mn-chloride and Cd-chloride (each 0.04%) are more or less better to produce this pigment.

This pigment is easily soluble in ether, methyl alcohol, ethyl alcohol, acetone, chloroform but not easily in benzene, petroleum ether and water. When the aqueous solution of this pigment is added with ammonia, fluorescence appears, but by addition of acid it disappears.

VI. Conclusion.

1. The fermentation products from glucose and saccharose by *Aspergillus glaucus* was studied.

2. Fumaric, citric, succinic, oxalic, tartaric and malic acids were isolated and identified.

3. Beside these acids, 2 new substances were isolated. And it was identified that one of them was 2-oxymethyl furane-5-carbonic acid.

4. Non-volatile acid was not produced but nearly trace of ethyl alcohol was produced.

5. The effects of nitrogen source and metallic salts on the pigment similar to fluorescein were studied but this pigment was not isolated as a crystal.

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