INFLUENCES OF HYDROGEN-ACCEPTOR ON FUCOSE FERMENTATION BY ASPERGILLUS ORYZAE.

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On the mechanism of fucose fermentation, no experimental results have been hitherto reported. It has to be explained by reference to pentose fermentation which has been investigated by some authors. The mechanism of pentose fermentation by fungus is different from that by bacteria. In the latter case, CO_2, acetone, acetic, lactic, and succinic acids, and ethyl alcohol are found in the fermentation products. In the former case it was stated by Behren(1) that *Botrytis cinerea*, *Rhizopus nigricans*, and *Penicillium glaucum* utilized arabinose. It was also stated by Czapek(2) that *Aspergillus niger* consumed xylose and arabinose, though the products are far different from those of fermentation by fungus. Niktinsky reported that *Aspergillus niger* easily utilized arabinose as a carbon source and that there is no difference from *d*-glucose. Peterson, Fred, and Schmidt(3) stated that when pentose, i.e. arabinose, was fermented by *Aspergillus*, by *Penicillium*, or by *Mucor*, they could not find any alcohol nor volatile acid excepting CO_2. White and Willamen(4) reported that *Fusarium* formed alcohol from arabinose. Amelung(5) isolated citric acid from *Aspergillus* culture media containing arabinose. Butkewitsch(6) stated that citric acid was formed from arabinose by *Citromyces* while only a trace was formed by *Aspergillus*. Accordingly the present author has explained the mechanism of arabinose fermentation as follows: arabinose would be broken down to C_3-compound at first and glucose might be changed to glucuronic acid then to saccharic acid and at last to citric acid. Jurist(7) stated that the saccharic acid then changes to ketipinic acid and at last to citric acid while Franzen and Schmidt formed citric acid from ketipinic acid ester, treating with KOH. Tamiya(8) found kojinic acid in the fermentation by *Aspergillus flavus*. Schreyer(9) reported that *Aspergillus*

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(1) Behren, *Centr. Bakt.*, II, **4** (1898), 514.

(2) Czapek, *Hofmeister's Beitrage*, **3** (1902), 62.

(3) Peterson, Fred, and Schmidt, *J. Biol. Chem.*, **60** (1924), 628.

(4) White and Willamen, *Biochem. J.*, **22** (1928), 583.

(5) Amelung, *Z. physiol. Chem.*, **166** (1927), 161.

(6) Butkewitsch, *Biochem. Z.*, **142** (1923), 195.

(7) Jurist, *J. Am. Chem. Soc.*, **44** (1925), 1136.

(8) Tamiya, *Acta Phytochim.*, **4** (1929), 227.

(9) Schreyer, *Biochem. Z.*, **202** (1928), 131.

formed fumaric acid from arabinose. There is only one report that methylpentose, i.e. isorhodeose, was fermented by *Aspergillus niger* to form tartaric acid.

Among the yeasts, it was reported by Lintner that *Schizosaccharomyces thermantitonum* ferments arabinose but other kinds of yeast do not. Many yeasts contain large quantities of methylpentosan, which is called yeast gummy substance and the nucleic acid of yeast contains ribose, a kind of pentose.

As to bacteria, it was reported by Schmidt and Peterson(10) that the acetone fermentation bacteria formed butyl alcohol, acetone, volatile and non-volatile acids with CO_2 and by Bertrand(11) that in sorbose fermentation, *Bacterium xylinum* formed arabonic acid. Lafar(12) stated that oxalic acid fermentation was caused by *B. aceti*, *B. acetogenum*, *B. ascendens*, *B. Kutzengianum*, *B. xylinum*, and by *Thermobacter aceti*. Grimbert(13) proved that *Pneumococcus* formed lactic acid and acetic acid from arabinose, Further, Tollens(14) proved that ethyl alcohol is formed by *B. ethaceticus* and by other putrefaction bacteria. Seiler(15) stated that anhydropentose was changed to galactan, $C_{12}H_{20}O_{10}$, by *B. metarabinum* and by *B. persiae*.

Among the references on the influences of the hydrogen acceptor, i.e. oxygen, methyl blue, and quinone, in acetic acid fermentation, Tamiya and Tanaka(16) stated that quinone is the most powerful reagent. Thus the dehydration due to the presence of these reagents in acetic acid fermentation is explained by the following chemical reaction: $CH_3CH_2OH + Acc. = CH_3CHO + H_2Acc.$ or $CH_3CH(OH)_2 + Acc. = CH_3COOH + H_2Acc.$ Also the same reaction was observed in the case of the formation of succinic acid from acetic acid, of fumaric acid and of succinic acid from alcohol and of fumaric acid from succinic acid. Fischer(17) observed fumaric and maleic acid formation from Na-succinate of muscle in the presence of methyl blue. Also there is need of the hydrogen acceptor in the formation of propionic acid from lactic acid and formic acid decomposition to CO_2.

However, there is no report concerning the products of fucose fermentation by *Aspergillus oryzae* nor the differences in the fermentation products when cultured by the same fungus under different conditions of culture.

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(10) Schmidt and Peterson, *J. Biol. Chem.*, **61** (1924), 163.

(11) Bertrand, *Compt. rend.*, **126** (1898), 762.

(12) Lafar, "Handbuch Tech. Mykolog.", Bd. 5, 588.

(13) Grimbert, *Ann. inst. Pasteur*, **9** (1895), 240.

(14) Tollens, *J. Landw.*, **49** (1901), 29.

(15) Seiler, *Centr. Bakt.*, II, **4** (1898), 514.

(16) Tamiya and Tanaka, *Acta Phytochim.*, **6** (1932), 1.

(17) Fischer, *Arch. Pharma.*, **257** (1919), 33.

The writer has had the opportunity of getting plentiful amounts of substances consisting principally of fucose in the experiments relating with chemical studies of *Iridaea laminarioides*.

Preparation of fucose. The air-dried samples of *Iridaea laminarioides*, upon extraction with 35% alcohol at 40°C., yielded a very small amount of viscous liquid containing colouring matters. The evaporated extract was mixed with acetone and the precipitate was freed from acetone and extracted again with alcohol to free it from chromoproteid. The residue was hydrolysed with 5% H_2SO_4, neutralised with $Ba(OH)_2$ and then filtered. The filtrate was evaporated and treated with alcohol to obtain the syrup of sugars. This syrup is made up of the hydrolytic products of the dilute alcohol soluble polysaccharides and the hydrolysed products are composed from fucose. *Aspergillus oryzae* was then cultured at 30°C. for a few days until the time of spore formation. The nutritional salts were prepared as follows: $NaNO_3 = 4$, $KH_2PO_4 = 2$, $MgSO_4 \cdot 7H_2O = 1$, $FeCl_3 = 1$ drop in 100 c.c. of water.

Separation and detection of the fermentation products. The fungus membrane of the surface of the fermented liquid was taken away, the liquid was filtered, and freed from volatile matters by steam distillation. The distillate was used as samples for the detection of acetaldehyde, acetone, alcohol, acetic acid, and formic acid.

The residual liquid was neutralised with NaOH, Cu-acetate was added to precipitate kojinic acid, the liquid filtered, and the filtrate was acidified with acetic acid and then precipitated with Pb-acetate. The Pb-precipitate may contain oxalic, citric, fumaric, tartaric, and allied acids, and when the filtrate is made alkaline with ammonia, the precipitate which is formed by Pb-acetate may contain glycolic acid, and its filtrate, lactic acid. The Pb-precipitate, containing oxalic, citric, and allied acids, was treated with H_2S and reprecipitated with Pb-acetate in 50% alcohol solution in the presence of ammonia. The oxalic acid fraction was insoluble and the citric acid fraction became soluble. The Pb-precipitate, containing citric and tartaric acid fraction was treated with H_2S; then the filtrate was concentrated and mixed with two volumes of 95% alcohol, some K-acetate was added. Then the precipitate was found to be composed of tartaric and the filtrate was citric acid.

For the detection of acetaldehyde, the silver mirror reaction, the phenylhydrazine reaction and the *p*-bromo-phenyhydrazine reaction were employed. For the detection of acetone, Gablielsen's Na-nitroprusside reaction was used with ammonia. For the detection of alcohol, the iodine and K-iodide reactions were carried out with KOH. For the detection of acetic acid, the formation of the hexa-acetate of ferri-salt was used, and also the distillation test of

Ca-acetate and the silver acetate crystals with $AgCO_3$. For the detection of formic acid, the precipitate by Pb-acetate in ammoniacal solution, the colour reaction with $FeCl_3$ and with resorcinol and H_2SO_4, and the detection of methyl blue with K-bisulphite were employed. For the detection of kojinic acid use was made of the colour reaction with $FeCl_3$, the reducing test with Fehling's solution, the diazo-reaction with diazobenzenesulphonic acid, and determination of the melting point. For the detection of lactic acid, the guaiacol reaction with H_2SO_4, the acetaldehyde formation, treatment with K-permanganate and the crystallisation of Zn-lactate were used. For the detection of glycolic acid, the *p*-cresol reaction with acetic acid and H_2SO_4, Bülow's hydrazine reaction with $FeCl_3$ and H_2SO_4, and the crystals and the melting point of the phenylhydrazide were examined. For the detection of citric acid, the formation of Hg-$HgSO_4$, acetone-dicarboxylic acid salt by Denigès's reagent, Stahre's reaction with K-permanganate, and Na-nitroprusside reaction were tested. For the detection of tartaric acid, Fenton's dihydroxymaleic acid reaction, the colour reactions with resorcinol, with α-naphthol and with ammonium molybdate were employed. For the detection of fumaric acid, the melting point of free acid and Ca-fumarate was examined. For the detection of oxalic acid, the colour reaction with $FeCl_3$, with resorcinol and H_2SO_4, and the Ca-Ag-oxalate were examined. For the detection of glycerinaldehyde, the orcinol and the phloroglucinol reaction, the reduction of Fehling's solution, and the phenylosazone were examined. For the detection of carboxylic acid, the colour reactions of α-naphthol, of phloroglucinol, of orcinol, of resorcinol and of naphthoresorcinol were examined, and the osazone test was made.

Differences between fucose fermentation products by *Aspergillus oryzae* with and without methyl blue. The liquid resulting from fermentation of fucose by *Aspergillus oryzae* with and without methyl blue, was filtered, and distilled in steam. Both the distillates showed neither the acetaldehyde reactions, i.e. the phenylhydrazine or the silver mirror reaction, nor acetone reaction, i.e. reaction with Na-nitroprusside, acetic acid, and ammonia. The filtrate showed also no alcohol reaction, i.e. reaction with KI, iodine, and KOH, and no acetic acid reaction, i.e. with $FeCl_3$. These results coincide with the results of tests of the distillate of arabinose fermentation which were reported in the Journal of Agr. Chemical Society of Japan.(18)

As to the detection of formic acid by means of Pb-acetate, and $FeCl_3$, and by resorcinol and H_2SO_4, the reactions were faint, and in the presence of methyl blue, these reactions were not observed at all, while as to the arabinose fermentation, pretty noticeable reactions were observed.

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(18) Tadokoro, *J. Agr. Chem. Soc. Japan*, **11** (1935), 167.

The distillation residue was neutralized with NaOH and a precipitate was formed, Cu-acetate added, the liquid filtered, the filtrate was freed from Cu-salts, and the Pb-precipitate was formed by Pb-acetate after acidification with acetic acid. In the detection of kojinic acid obtained from H_2S decomposition of Cu-precipitates, the $FeCl_3$ reaction, the reduction of Fehling's solution, and the diazo-reaction, were positive, although, when it was extracted with ether, no crystals could be obtained ; but when methyl blue was used, no reaction could be observed.

The Pb-precipitate which corresponds to the oxalic and citric acid fraction, was obtained as stated above by the treatment with Pb-acetate and acetic acid. From the filtrate, after making it alkaline with ammonia, the Pb-precipitate was obtained, which corresponds to the glycolic acid fraction. After the separation of this precipitate, the filtrate contained also Pb-salts which correspond to the lactic acid fraction.

The Pb-precipitate of the oxalic acid and citric acid fraction was decomposed with H_2S, and after being concentrated, Pb-acetate and ammonia were added in the presence of 50% alcohol. Therefore the oxalic acid fraction comes out as precipitate while the citric acid fraction remains in the filtrate. All these Pb-salts were decomposed with H_2S, filtered, the filtrate was concentrated and the various reactions were examined.

For the detection of glycolic acid, the concentrated solution was treated with ether. After the ether extract was freed from ether, the following reactions were tested. The cresol reaction with glacial acetic acid and H_2SO_4 showed greenish blue and the guaiacol reaction showed violet colour. Bülow's reaction⁽¹⁹⁾ with $FeCl_3$ and H_2SO_4 in the presence of phenylhydrazine showed reddish violet. The phyenylhydrazone was formed, treated with phenylhydrazine on the water bath, freed from the excess of phenylhydrazine, extracted with ether, and white crystals of the phenylhydrazide were obtained which melted at 118°C. after purification with acetic acid. But in the case of using methyl blue, these colour reactions were not observed at all.

For the detection of lactic acid and maleic acid, after the concentrated solution was treated with ether, examination was made of the following reaction.

As to the formation of acetaldehyde, upon heating the solution with K-permanganate it was detected. The rose colour was also observed after treating with H_2SO_4 and guaiactincture, and bright yellow was seen with $FeCl_3$. A large quantity of the liquid was boiled with $ZnCO_3$, and crystals of Zn-lactate were obtained. When the ZnO content was determined by the ordinary method, it was found to correspond to 28.02%. But in the case of

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(19) Bülow, *Ann.*, **236** (1886), 195.

using methyl blue, these reactions were not observed and naphthoresorcinol reaction only was seen. Further for the detection of maleic acid, Denigès's reaction⁽²⁰⁾ and diazo-reaction were observed, but Kleeman's reaction could not be observed. From these results in the case of using methyl blue, maleic acid is apparently formed instead of lactic acid.

For the detection of oxalic acid, the precipitate with $CaCl_2$ in ammoniacal solution which is insoluble in acetic acid was formed. The resorcinol H_2SO_4 reaction showed green blue, the $FeCl_3$ reaction showed greenish yellow. But Cu-oxalate with Cu-acetate, and Ag-oxalate with $AgNO_3$ were formed in small quantities in the presence of HNO_3, so the liquid does not seem to contain large quantities of oxalic acid.

In the detection of citric acid, the white precipitate with Denigès's reagent and the characteristic colour with Na-nitroprusside were not observed, so the presence of citric acid is questionable. For the detection of tartaric acid, Fenton's reaction with $FeSO_4$ and H_2O_2 and the ammonium molybdate reaction with H_2O_2 were negative. Therefore the presence of tartaric acid is also uncertain. The resorcinol H_2SO_4 and the α-naphthol-H_2SO_4 reactions which are common to this acid and succinic acid were observed. But the pyrrole-reaction of succinic acid was very faint, and in the $FeCl_3$ reaction no precipitate was formed, but the material showed only a clear red colour. From these results it may be stated that the presence of succinic acid is questionable while that of oxocarboxylic acid is more probable. Further, in the phloroglucinol-HCl and in the orcinol-HCl reactions, the red colour was indicated in amyl alcohol, and the reddish violet colour was indicated in ether in the naphtho-resorcinol-HCl reaction. The oxocarboxylic acid not precipitated by Cu-acetate, did not give the diazo-reaction and did not reduce Fehling's solution; those points are distinguished from kojinic acid. This acid formed osazone with phenylhydrazine and acetic acid, it is soluble in ether and melts at 155–158°, so it is near to the melting point of glucuronic acid. In the case of using methyl blue, it gave a strong diazo-reaction, so it is probable that maleic acid is formed in the fermented liquid.

The lactic acid fraction was extracted with ether, and the extract freed from ether. It showed red colour when tested with orcinol, with phloroglucinol and H_2SO_4, and also with resorcinol and HCl, so it seems to contain glycerinaldehyde.

Differences in chemical mechanism of fucose fermentations with and without methyl blue. Recently Peterson and Fred⁽²¹⁾ observed the formation of lactic and acetic acids in the heterogeneous lactic acid fermentation of

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(20) Denigès, *Compt. rend.*, **130** (1900), 32.
(21) Peterson and Fred, *J. Biol. Chem.*, **64** (1925), 643.

xylose by *Lactobacillus pentaaceticus* and also of arabinose by *Pneumococcus* as stated above. As it has already been stated by Virtanen(22) that the preform of lactic acid is methylglyoxal hydrate in sugar fermentation by *B. coli*, this mechanism will be assumed in the following methylpentose fermentation. Years ago Tates(23) stated that lactic acid formation was observed in the fermentation of rhamnose by *Friedlander's bacillus*, and the other report stated also that isorhodeose was fermented by certain microorganisms. In the case of methylpentose fermentation, the glycolic acid formation was observed always remarkably, so the chemical mechanism of other acid formation may be communicated to this acid. For example, it was stated by Challenger that formic acid was formed from glycolic acid in oxidative fermentation by *Aspergillus niger*.

Butkewitsch(24) stated that citric acid is formed always from glycolic acid by condensation. Fedorff proved the formic acid and the acetaldehyde formation from methylglyoxal by bacteria, and Sakaguchi proved that citric acid is formed easily from acetaldehyde by *Aspergillus* as had already been stated by Neuberg. Tamiya(16) stated that citric acid is formed from two mols of acetic acid and one mol of formic acid.

The acetaldehyde might be formed from methylglyoxal by the dehydration process. In the condensation of acetaldehyde, oxalacetic acid is formed at first, and then maleic acid might be formed also. The maleic acid formation in hexose fermentation was proved by Nishikawa, with cultured *Aspergillus*. Aubel(25) stated following [illegible]stel's explanation that by oxidation, propionic, acetic, and formic acids are formed while by dehydration, fumaric, maleic, and lactic acids are formed from succinic acid in sugar fermentation.

Sumiki(26) proved that the maleic acid formation with other organic acids occurs in the hexose fermentation by *Aspergillus glaucus*.

In the fucose fermentation, the presence of glycerinaldehyde was proved in the experiment as the intermediate product, so kojinic acid was assumed to be formed from glycerinaldehyde by the oxidative synthesis as stated by Tamiya, and the citric acid is formed by the condensation of glycerinaldehyde as stated by Sakaguchi.

The presence of oxocarboxylic acid in the sugar fermentation liquid was reported by Butkewitsch who stated that bacteria formed glucuronic acid from

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(22) Virtanen, *Ann. acad. sci. Fennicae*, **29** (1927), No. 26.

(23) Tates, Czapek's "Biochem. d. Pflanzen", I, 1913, 316.

(24) Butkewitsch, *Biochem. Z.*, **142** (1923), 195.

(25) Aubel, *Ergebnisse d. Enzymforschung*, **4** (1935), 251.

(26) Sumiki, *J. Agr. Chem. Soc. Japan*, **6** (1930), 1153; **7** (1931), 819.

gluconic acid. According to Sumiki the latter is proved in the oxidative fermentation of hexose by *Aspergillus*. In the same way, it was stated by Tamiya that arabonic acid is formed in arabinose fermentation by *Aspergillus flavus*, and also by Bertrand that the same acid formation is caused by *Bacterium xylinum*. Next, Sumiki(26) proved 2-hydroxymethylfurane-5-carboxylic acid formation in the hexose fermentation by *Aspergillus glaucus* and gluconic acid formation by other *Aspergillus*. Further Sakaguchi proved gluconic acid formation from glycerine by *Aspergillus*, so it shows that the gluconic acid formation is not only a result of the simple oxidation of glucose but it is caused by the oxidative synthesis from the substances with smaller molecules. Therefore, it is probably formed from pentose and methylpentose and also from their cleavage products. The author found glycerinaldehyde which comes easily from glycerine by dehydration in the fucose fermented liquid, and it is more likely that glycerinaldehyde forms gluconic acid and then oxocarboxylic acid.

Anyhow, the important fact of chemism in the cleavage of pentose molecule is the formation of C_3- and C_2-compound as stated by Lek(27) that in butyl alcohol fermentation by bacteria, the first cleavage of pentose molecule occurs to form an equimolecular mixture of methylglyoxal and glycoaldehyde.

In the above explanation, the author intended to consider the influences of the hydrogen acceptor in the light of the experimental results reported in the above section.

As described in the above section, the influences of methyl blue on acetic acid fermentation caused acetaldehyde to be formed from ethyleneglycol, etc. In muscle there is formed also maleic acid from succinic acid, etc. And it is known that the dehydration reaction of yeast(28) is greatly retarded in the presence of methyl blue and their cleavage mechanism is prevented, so much quantities of acetaldehyde were found instead of alcohol, caused by delay in the dismutation of acetaldehyde to alcohol. For the same reason, the action of yeast on methylglyoxal and on pyroracemic acid is always delayed. It is known that various changes were wrought upon chemism of fermentation by the presence of sulphite such as the accumulation of acetaldehyde in alcohol fermentation and in butyric acid fermentation, and such as that of pyroracemic acid in propionic acid fermentation of glucose.

In fucose fermentation with methyl blue, the formic acid formation was delayed, and this change of chemical mechanism is caused by the prevention of the oxidation and of the dehydration of these acid forming substances. So the prevention of the following chemical mechanisms happens as stated by

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(27) Lek, Diss. Delft, 1930.

(28) Sonderhoff, *Ergebnisse d. Enzymforschung*, **3** (1934), 183.

many authors in the different cases of fermentation, for instance, by Harden, by Grey, by Neuberg and by Scheffer in hexose fermentation by *B. coli*:

$$C_6H_{12}O_6+H_2O \rightarrow CH_3COOH+CH_3CH_2OH+2HCOOH \text{ (Harden)};$$
$$CH_3COCHO \rightarrow HCOOH+CH_3CHO \text{ (Grey)};$$
$$RCOCOOH+H_2O \rightarrow RCOOH+HCOOH \text{ (Neuberg)};$$
$$C_6H_{12}O_6 \rightarrow 2C_3H_6O_3,\ CH_3COCH(OH)_2 \rightarrow CH_3CHO+HCOOH \text{ (Scheffer)}.$$

The last reaction is also stated to occur in hexose fermentation by *B. Aerogenes*. Brinkmann stated the following reaction in anaerobic culture of lower fungus:

$$2CH_2COCOOH \rightarrow COOHCOCH_2CH_2COOH+2H_2O \rightarrow HCOOH+COOHCH_2CH_2COOH.$$

By Challenger the following reaction was stated in the culture media of *Aspergillus niger*: $CH_2OHCOOH \rightarrow HCOOH+H_2O+CO_2$.

Thus the formation of CO_2 and H_2 from formic acid is caused by dehydration, and these reactions must be delayed also as less CO_2-formation was observed in the experimental results.

Aubel stated that in the oxidative fermentation of hexose by *Aspergillus*, propionic, acetic, and formic acids were formed, while in the case of the prevention of oxidation, fumaric, maleic, and lactic acids were formed by dehydration and also maleic acid formation was observed from lactic acid. So in the experiment of the fucose fermentation, using methyl blue, maleic acid would be observed instead of formic acid which is formed in the case of fermentation without methyl blue. The following lactic acid formation is also prevented by using methyl blue in the fucose fermentation:

$$CH_3COCHO \rightarrow CH_3CHOHCOOH.$$

Glycolic acid is a normal product in the fucose fermentation by *Aspergillus oryzae* but it could not be detected under the influence of the addition of methyl blue, so the following reaction would be prevented:

$$CH_3COOH \rightarrow CH_2OHCOOH \rightarrow HCOOH+H_2O+CO_2;$$
$$CH_2OHCHO \rightarrow CH_2OHCOOH.$$