CCXLVI. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS.

XXXV. THE METABOLIC PRODUCTS OF *BYSSO- CHLAMYS FULVA* OLLIVER AND SMITH.

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OLLIVER AND SMITH [1933] described a new Ascomycete, *Byssochlamys fulva*, which is of fairly frequent occurrence as a cause of spoilage in processed fruits and is of considerable economic importance in the fruit canning industry. The source of the fungus is at present unknown, and the prevention of infection is difficult on account of the high resistance to heat shown by the spores. The mature conidia are unaffected by the normal sterilising process in which the maximum temperature attained exceeds 90°. In connection with its resistance to what would usually be considered lethal conditions it is of interest to note that the type species of the genus, *B. nivea* Westling [1909], can withstand prolonged immersion in 90 % alcohol and was actually isolated from botanical specimens preserved in spirit, the mould appearing as soon as a portion of the specimen became uncovered owing to gradual evaporation of the preservative.

The present paper is an account of the chief metabolic products which are formed when *B. fulva* is grown on a synthetic medium containing glucose as the sole source of carbon. In a preliminary test, in which the mould was grown on Czapek-Dox solution in test-tubes, it was noted that, after about 4 weeks' incubation, addition of mineral acid to the metabolism solution gave a precipitate which was soluble in ether. Further experiments show that the amount of precipitate obtained increases with continued incubation and appears to reach a maximum when the glucose is entirely consumed; also that the yield varies with the temperature of incubation, being less at 30° than at 24° and becoming nil at 37°. Large-scale experiments, in which the mould was grown on batches of 35 litres of medium at 24°, have resulted in the isolation, from the acid precipitate, of a new and specific mould product, $C_{18}H_{20}O_8$, M.P. 163·5°, which is definitely toxic to mice and for which the name *byssochlamic acid* is proposed. The substance titrates as a tetrabasic acid, giving salts of the type $C_{18}H_{20}O_8R_4$, and it is probable that it is contained in the metabolism solution as a mixture of salts of this general formula, acidification giving the free acid, $C_{18}H_{24}O_8$, which immediately loses two molecules of water to give $C_{18}H_{20}O_8$. It is interesting to note that Wijkman [1931] describes a mould product, glauconic acid II, which has the formula $C_{18}H_{20}O_8$ and titrates, on warming, as a tetrabasic acid. The melting-point of glauconic acid II, however, 186°, is sufficiently different from that of our product, 163·5°, to warrant our presumption that the two are different substances.

The main metabolic product of the mould is mannitol, formed in amounts corresponding roughly with 30 % of the total sugar utilised. Other substances are produced in small amounts and are precipitated along with the byssochlamic acid, but these have not yet been investigated. This is only one of many instances of the production of mannitol from glucose by mould fungi. It is now known that a fairly large number of species, belonging to several different genera (*Penicillium*, *Aspergillus*, *Helminthosporium*, *Clasterosporium*, and now *Byssochlamys*) can effect this conversion. It is certainly remarkable that the production of mannitol from glucose is so common, whilst there is no known case of the conversion by moulds of glucose into sorbitol, a change which is easy to effect by purely chemical reduction.

EXPERIMENTAL.

The culture medium used was the standard Czapek-Dox solution containing 5 % of glucose: water to 1000 cc.; glucose, 50 g.; NaNO_3 , 2 g.; KH_2PO_4 , 1 g.; KCl , 0.5 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g.; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g. Five large-scale experiments have been carried out, in each case using 100 conical flasks, of 1-litre capacity, each flask containing 350 cc. of solution. The medium was sterilised by steaming for an hour on each of three consecutive days and was then sown with a spore suspension made from 6 cultures of *B. fulva* on wort-agar slopes, the cultures being at least 10 days old.

In the first experiment three of the flasks were incubated at 37°, three at 30° and the remainder at 24°. At 37° growth was exceedingly slow at first owing to the fact that the spores used for inoculation were completely wetted and sank to the bottom of the liquid, with the result that a gelatinous, submerged mycelium was slowly formed, and only when this was sufficiently extensive to reach the surface were normal aerial colonies produced. After this, growth was rapid, but at no stage did the solution give a precipitate on addition of acid. The results of analyses of the contents of flasks incubated at 30° and 24° are given in Table I. The column headed "On acidification" indicates the result

Table I.

Days incubation	30°			24°		
	p_{H}	% glucose by polarimeter	On acidification	p_{H}	% glucose by polarimeter	On acidification
18	4.5	3.87	Solution clear	4.7	4.33	Solution clear
32	4.5	1.61	Faint opalescence	4.8	2.26	Slight precipitate
57	4.5	Nil	Very slight precipitate	4.8	Nil	Considerable precipitate

obtained by making the filtered metabolism solution acid to Congo red by addition of HCl , when the appearance of the liquid is a rough indication of the amount of the specific product. The sugar is thus utilised more rapidly at 30° than at 24° (this is confirmed by the growth rate as judged visually), but less of the specific product is formed.

The contents of the remaining 91 flasks were worked up, as described below, for isolation of products at the end of 57 days' incubation.

In the second and subsequent experiments all the flasks were incubated at 24° until the solution had a rotation close to zero, the periods varying from 56 to 63 days. At the end of the incubation period the contents of the flasks were filtered, the mycelium was well squeezed out and washed with water, and

an average sample of the mixed filtrate and washings taken for analysis. To the main bulk of liquid was added, with vigorous stirring, sufficient concentrated HCl to make the liquid definitely acid to Congo red (100 cc. acid). A yellow flocculent precipitate formed at once and, after standing overnight, was filtered off, washed with water and dried *in vacuo* over H_2SO_4 (acid precipitate, see below). In no case did further addition of acid to the mother-liquor give any more precipitate.

In Exp. 1, the mother-liquor was neutralised to methyl red by 450 cc. of 2*N* NaOH and then evaporated to 800 cc. *in vacuo* at 45–50°. On cooling, the concentrate deposited a large amount of semi-crystalline solid and, on standing at 0°, a further quantity of solid separated in the form of long, colourless needles. A small amount of the latter was taken out, drained at the pump and cautiously washed with cold water till free from the brown mother-liquor. The crystals were perfectly colourless, dissolved readily in cold water, had m.p. 166–167° and, mixed with a sample of pure mannitol, melted at 166–167°. By recrystallisation from 66 % alcohol of the main bulk of solid matter the latter was shown to consist almost entirely of mannitol mixed with small amounts of inorganic salts.

In Exps. 2, 3 and 4 mannitol was estimated in the filtered metabolism solution, using the polarimetric method of Raistrick and Young [1931] (see Table III, column 5). The total volume of solution in each case was measured and the total production of mannitol from the 1750 g. of glucose originally present was calculated. In Exp. 4 the metabolism solution, after removal of the acid precipitate, was concentrated *in vacuo* (without previous neutralisation), the separated solid recrystallised from 66 % alcohol and the mother-liquor alternately concentrated and treated with 2 volumes of alcohol until no further mannitol could be obtained. The total recovery of mannitol, attained without any preliminary purification of the solution, was 80 % of the estimated amount.

Treatment of the acid precipitate. In Exp. 1 the dry solid was yellowish-brown in colour and weighed 18.1 g. It was extracted with ether in a Soxhlet apparatus until nothing further was dissolved. Crystals soon began to separate in the extraction flask and gradually a dirty green solid, slowly turning black, was deposited above the liquid level, the ether solution being reddish-brown. The extraction was stopped at intervals, the crystalline solid being filtered off, well washed with cold ether and dried in air. The total time of extraction was approximately 16 hours, and three solid fractions were obtained. A further quantity of solid was obtained by concentration of the combined mother-liquors, after which further evaporation gave only a thick brown syrup. The characteristics of the four solid fractions are given in Table II. Mixtures of any of the fractions

Table II.

Fraction	Colour	Weight g.	M.P.
<i>a</i>	Faint yellow	1.70	163°
<i>b</i>	Pale bluish grey	4.15	162–163°
<i>c</i>	Dark grey	1.70	162–163°
<i>d</i>	Faint yellow	0.48	163°

all melted at 162–163°, showing that all consisted essentially of the same substance. From the mycelium of the fungus a further quantity of identical material was obtained. The mycelium was dried *in vacuo* at a low temperature, finely powdered and extracted first with light petroleum, which rapidly removed a quantity of dark brown sticky material. Subsequent extraction with ether gave

1.08 g. of almost colourless prisms, M.P. 162.5–163°, unchanged by mixing with fraction "a" (above).

The crude crystalline material was purified as follows. It was dissolved in benzene (approximately 6 cc. to every 1 g. of substance), boiled for some time to coagulate traces of the greenish pigment, the solution filtered hot and mixed with twice its volume of boiling ether. On cooling, colourless prisms, M.P. 163°, separated in almost quantitative yield.

The acid precipitates obtained in Exps. 2–5 had similar characteristics and were treated in the same way.

The yields of metabolic products, and other experimental details, are given in Table III.

Table III.

Experi- ment number	Incubation period days	Metabolism solution		Mannitol		Wt. of acid precipitate g.	Wt. of byssochlamic acid g.
		Volume litres	Rotation 2 dm. tube Hg ₅₄₆₁	Estimated g.	Isolated g.		
1	57	—	Nil	—	—	18.1	8.03
2	63	31.8	+ 0.23°	597	—	16.8	6.28
3	56	31.35	+ 0.025°	498	—	20.1	7.20
4	56	31.48	− 0.03°	460	366	19.8	6.13
5	59	—	− 0.06°	—	—	23.5	8.10

Characterisation of mannitol. All the mannitol isolated was in the form of colourless needles, M.P. 166°, and, mixed with an authentic sample of pure mannitol, had M.P. 166–167°. In a 2 dm. tube the rotation of a 2 % solution was $\alpha_{5461}^{18^\circ} - 0.02^\circ$, whilst a 2 % solution in a 6 % borax solution gave, in a 4 dm. tube, $\alpha_{5461}^{18^\circ} + 2.92^\circ$ and $\alpha_{5790}^{18^\circ} + 2.56^\circ$. The mean percentage of mannitol in the solution, calculated from the rotations using the tables of Raistrick and Young [1931], is 2.0.

Byssochlamic acid. Byssochlamic acid was obtained by crystallisation from a mixture of ether and benzene in the form of colourless prisms, M.P. 163°. Recrystallisation from alcohol gave crystals of the same form, M.P. 163.5°, and the melting-point was not raised by further recrystallisation from alcohol, or benzene and light petroleum. There is no obvious decomposition at the melting-point, and the melt is clear and colourless but does not resolidify on cooling, even when seeded. The pure substance is insoluble in water. It is slightly soluble in alcohol, the solution being faintly acid to litmus and giving no colour reaction with FeCl₃. Its solubilities, at room temperature, in various organic solvents, expressed as g. substance in 100 cc. of solvent are approximately: acetone, 63; ethyl acetate, 33; chloroform, 20; benzene, 7.5; ether, 0.33; alcohol, 0.3; light petroleum less than 0.1. By slow evaporation of solutions in acetone or benzene the substance may be obtained in beautiful rectangular prisms up to half-an-inch long. Byssochlamic acid is insoluble in cold dilute NaOH, but, on heating, dissolves slowly. It is reprecipitated unchanged by addition of mineral acid to the alkaline solution. It dissolves readily in warm concentrated HNO₃ and is recovered unchanged on evaporation of the solution. It is soluble in warm concentrated H₂SO₄ and is reprecipitated on addition of water. The H₂SO₄ solution darkens slowly when strongly heated.

Micro-analysis (Schoeller) gave: Sample 1: C, 65.11, 65.16 %; H, 6.12, 6.16 %; N, negative; OCH₃, negative; molecular weight, 310, 326. Sample 2: C, 65.15, 65.04 %; H, 6.10, 5.99 %. C₁₈H₂₀O₆ requires C, 65.03 %; H, 6.07 %; molecular weight, 332.

Estimations of molecular weight by depression of the freezing-point of benzene gave somewhat erratic results. In a 0.7 % freshly made solution, the observed molecular weight was 586. This fell gradually on standing to a final steady value of 322. In 1.3 % solution the final value was 352, and in a 2 % solution 407. The lowest values obtained indicate that the correct formula is $C_{18}H_{20}O_8$ and not $C_9H_{10}O_3$.

Titration with $N/10$ NaOH to phenolphthalein in aqueous solution can be carried out only if the temperature is maintained near the boiling-point. Even then solution proceeds very slowly but the end-point is quite sharp. 0.1973 g. substance required 23.62 cc. $N/10$ NaOH, whence the equivalent (E) is 83.5. In acetone solution titration to phenolphthalein proceeds smoothly in the cold and without lag. 0.2003 g. of substance, dissolved in 25 cc. of pure acetone, required a net amount, corrected for blank, of 24.01 cc. $N/10$ NaOH, whence $E = 83.4$. $C_{18}H_{20}O_8$ titrating as a tetrabasic acid has $E = 83.04$.

The specific rotation, using a 0.4 % solution in $CHCl_3$, is $[\alpha]_{5461}^{20^\circ} + 127^\circ$, $[\alpha]_{5780}^{20^\circ} + 108^\circ$.

The sodium salt is extremely soluble in water. Evaporation of the solution at room temperature *in vacuo* gives a syrup which slowly solidifies in micro-crystalline form. A 1 % solution of the sodium salt was used to test reactions with solutions of various metallic salts. Each test was made on 2 cc. of solution, the appropriate reagent being added drop by drop until no further reaction occurred, with the following results.

$AgNO_3$ gives a white, amorphous precipitate, readily soluble in dilute HNO_3 or NH_4OH , unchanged on standing for 24 hours but darkening gradually after several days or on prolonged heating at 100° . $BaCl_2$ gives a white precipitate separating slowly as rosettes of microscopic needles, and $FeCl_3$ a heavy, buff precipitate insoluble in excess of the reagent. Both normal and basic lead acetates give heavy, white amorphous precipitates. $CaCl_2$ gives no precipitate even on addition of four volumes of alcohol. Salts of Hg, Cu, Ni and U give no precipitates but, with the exception of Hg, give solutions which are much more intensely coloured than the reagents used.

A quantity of the silver salt was prepared by adding approximately 100 % excess of the theoretical amount of $AgNO_3$ to a neutral solution of the sodium salt, the white amorphous precipitate being filtered off, washed and dried at 100° . By ignition, Ag, 54.11, 54.10 %. Calculated for $C_{18}H_{20}O_8Ag_4$; Ag, 54.23 %.

An unsuccessful attempt was made to prepare the free acid $C_{18}H_{24}O_8$ by cautious acidification of the sodium salt. 0.5193 g. of byssochlamic acid was dissolved by heating with 62.5 cc. of $N/10$ NaOH. The cooled solution was covered with 200 cc. of ether, and 59.5 cc. of $N/10$ H_2SO_4 (approximately 5 % less than the theoretical amount) were added in 2 cc. portions, shaking vigorously between additions. The ether solution was evaporated at room temperature *in vacuo* and the white residue dried by exposure to air. The M.P. was 163° , with no visible change at any lower temperature. A sample heated to constant weight at 100° lost 1.1 %, the calculated loss, from $C_{18}H_{24}O_8$ changing to $C_{18}H_{20}O_8$, being 9.8 %.

Toxicity of byssochlamic acid. Toxicity tests have very kindly been carried out for us by Prof. Topley of the London School of Hygiene and Tropical Medicine. Twelve mice in three groups of four were injected with a solution of the readily soluble sodium salt. Three solutions in normal saline were made, such that the required amounts of substance were contained in 0.5 cc. The mice in the first group each received an intraperitoneal injection of 25 mg. of the sodium salt, and all were dead in 24 hours. The second group received 12.5 mg.

and, of these, one died in 24 hours, two after 2 days and one after 3 days. The third group were injected with 6.25 mg.; one died in 2 days, two in 3 days and one in 6 days. The substance thus shows a definite, though low, toxicity to mice.

Further work is in progress with the object of elucidating the molecular structure of byssochlamic acid and will be reported in due course.

SUMMARY.

1. *Byssochlamys fulva* Olliver and Smith has been grown on Czapek-Dox solution at 24°, and it is shown that the metabolic product formed in greatest amount is mannitol. Yields equivalent to 30 % of the sugar consumed were obtained.

2. A new mould product, byssochlamic acid, $C_{18}H_{20}O_6$, M.P. 163.5°, has also been isolated from the metabolism solution, in yields of about 0.5 %. Byssochlamic acid is toxic to mice.

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