FUNGAL CELLULASES

III. *STACHYBOTRYS ATRA*: GROWTH AND ENZYME PRODUCTION ON NON-CELLULOSIC SUBSTRATES

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Summary

The growth of the mould *Stachybotrys atra* on modified Waksman-Carey medium has been studied. Iron, zinc, copper, and manganese have been shown to be inorganic micronutrients for the mould but their essential nature has not been conclusively demonstrated. Certain other supplements appear to stimulate growth.

When ammonia is the nitrogen source, the mould makes good growth only with sugars and related compounds as carbon sources; the only other carbon source readily utilized is acetate. Replacement of ammonia by nitrate assists the utilization of a range of organic acids. A wide range of nitrogen sources can be utilized for growth; proteins are good sources of both carbon and nitrogen, but individual amino acids are inadequate.

The course of growth on sucrose, starch, and both buffered and unbuffered glucose media has been followed. Enzymes with β-glucosidase activities were produced on all media and the presence of substrates containing β-glucosidic linkages was unnecessary for the production of activity simulating the "C_x enzyme" (enzyme splitting polymeric β-glucosidic linkages) of Reese, Siu, and Levinson (1949).

High activity for the hydrolysis of salicin and sodium carboxymethyl cellulose appears in the medium at and just after the germination of the mould. In unbuffered media a second maximum is associated with the attainment of maximum mycelial weight. Enzymic activity against *p*-nitrophenyl-β-glucoside does not appear to be closely correlated with the other two activities. The meaning of these results is discussed in terms of the hypothesis of multiple β-glucosidase activities and the non-existence of a specific "C_x" enzyme (Jermyn 1952*b*).

I. INTRODUCTION

Stachybotrys atra Corda is typically north temperate in its distribution according to White, Yeager, and Shotts (1949) but is also a very frequent isolate in the Melbourne area (Cox *et al.* 1945). In tropical areas *S. atra* is replaced by the very closely related *Memnoniella echinata* (Riv.) Gallow. The complete elimination of invalid species in *Stachybotrys* has not yet been accomplished and Bisby (1945) believes that many of the species described will eventually be reduced to synonymy in the highly variable *S. atra*. The uncertainty about identifications in this genus must be borne in mind in comparing the various physiological studies in the literature. Siu (1951) classifies *S. atra* among the "strong" cellulolytic fungi with abundant isolations from decomposing cellulosic

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materials in the field; the "very strong" *Myrothecium verrucaria* on which the majority of studies on fungal cellulase have been made in the past few years (Saunders, Siu, and Genest 1948; Whitaker 1951) seems unable to maintain itself on cellulosic substrates in the field. For this reason S. *atra* has been selected for fundamental studies in an Australian laboratory on the production of cellulolytic enzymes by microorganisms.

Reese, Siu, and Levinson (1949) have postulated that two types of enzymes are required for the breakdown of cellulose. The first ("C_1 enzyme"), which is present only in cellulolytic fungi, degrades native cellulose to a form that can be further hydrolysed by the second ("C_x enzyme") which is present in all cellulolytic and most non-cellulolytic fungi. It has been shown (Jermyn 1952*a*, 1952*b*) that the non-cellulolytic fungus *Aspergillus oryzae* releases C_x activity into the culture medium and that this activity is due to the presence of some eight β-glucosidases, which have different degrees of specificity for different substrates. Production of these enzymes took place in the absence from the culture medium of substrates having β-glucosidic linkages.

The work presented in this paper is designed to test whether the "C_x" activity of S. *atra* is produced in the culture medium in the absence of β-glucosidic carbon sources and to discover conditions for producing the enzyme or enzymes involved in this activity in sufficient concentrations to warrant purification. It also extends the results of Perlman (1948) to give a wide enough preliminary survey of the growth of S. *atra* in shake culture to serve as a basis for a programme of physiological research on the fungus.

II. Cultural Conditions

To simplify this preliminary study only certain of the possible variables in culture conditions have been investigated. Except where specific mention of the fact is made, the conditions specified in the description below were used throughout.

Incubation was carried out in a warm room maintained at 28 ± 0.5°C., which is approximately the optimum temperature for growth of S. *atra* (Basu 1948). The shaker used was adapted from the design of Jennison *et al.* (1950); it had a reciprocating action with a total excursion of 2 in. and a speed of 96 cycles per minute. Where a continuous record of the pH of the culture medium was to be taken, a Leeds and Northrup Micromax recording pH meter with shielded leads was used. It was found that the directly determined pH of the culture medium and that recorded on the chart were in agreement for runs of up to 3 weeks.

A culture of S. *atra* was obtained from Mr. F. M. Crook of the Defence Research Laboratories, Melbourne. It agreed in all particulars with the literature descriptions of the species. The fungus was maintained by serial transfer on potato-dextrose sugar slopes. After 10 days growth at 30°C. tubes were kept in a refrigerator at 5°C. till required. A spore suspension from the tubes was used to inoculate a layer of potato-dextrose agar medium in 250 ml. Roux bottles. After 10-14 days incubation at 28°C., the contents of the Roux bottle were used to prepare spore suspensions for inoculating the culture flasks.

The basic mineral medium used throughout was that of Waksman and Carey (1926) as modified by Fahraeus (1947). In accordance with the findings of Marsh and Bollenbacher (1946), Perlman (1948), and Buston and Basu (1948), 12 μg. of biotin was routinely added per litre of medium. After experiments on mineral nutrition had indicated the stimulating effect of zinc ions, the Waksman-Carey medium was further modified by the addition of 2 mg./l. of $ZnSO_4.7H_2O$. The final composition of the medium was as follows:

	g./l.
$(NH_4)_2HPO_4$	2.5
$MgSO_4.7H_2O$	0.5
KCl	0.5
$CaCl_2$	20×10^{-3}
$FeSO_4.7H_2O$	10×10^{-3}
$ZnSO_4.7H_2O$	2×10^{-3}
$MnSO_4.4H_2O$	1×10^{-3}
D-biotin	12×10^{-6}

Using this medium experiments were performed to test:

(i) *Ability of* S. atra *to Use Various Carbon and Nitrogen Sources.*—The medium was distributed into 250-ml. conical flasks, 50 ml. being added per flask and, after autoclaving, 0.2 ml. of spore suspension containing 10^9 spores/ml. was added to each.

(ii) *Enzyme Production by* S. atra *on Various Media.*—A three-necked, 2-l. Woulffe bottle containing 500 ml. of medium was autoclaved, one of the necks being fitted with a potassium chloride bridge extension from a calomel electrode dipping into the liquid. On cooling, an extended glass electrode, sterilized in distilled water, was introduced into a second neck. The contents of the bottle were then inoculated and the electrodes connected to the recording pH meter. Samples were withdrawn as required through the third neck of the bottle.

A continuous record of mycelial weight could not be obtained from this apparatus; a set of 250-ml. flasks was therefore inoculated in parallel groups of four flasks chosen by lot removed from the shaker from time to time, and the bulked mycelial weight calculated to give that from 500 ml. of medium. Growth in the flasks was in general a little ahead of that in the bottle and Figures 3-8 must be interpreted with this fact in mind.

III. Determination of Enzymes and other Components of the Culture Medium

(*a*) C_x *Enzyme and Other β-Glucosidases*

The procedure for the C_x enzyme using sodium carboxymethyl cellulose as substrate has been outlined by Jermyn (1952*a*). A similar procedure with 1 per cent. solution of sodium cellulose sulphate (Eastman Kodak Co.) as substrate was also used.

The measurement of salicinase and *p*-nitrophenyl-β-glucosidase has also been outlined by Jermyn (1952*a*).

(*b*) *Amylase and Sucrase*

The procedure of Crewther and Lennox (1952) was used.

(*c*) *Proteases*

The activity of enzyme solutions in reducing the viscosity of gelatin was measured according to the method of Lennox and Ellis (1945). Their action on gelatin was also measured by the gravimetric technique of Crewther (1952). These are the "viscometric" and "gravimetric" enzymes of Crewther and Lennox (1953).

TABLE 1

GROWTH OF *S. ATRA* ON ACETATE MEDIA, CONTAINING THE SAME MINERAL ELEMENTS AS THE WAKSMAN-CAREY MEDIUM AND FURTHER SUPPLEMENTED BY THE ADDITION OF POSSIBLE MICRONUTRIENTS

Initial pH 7.0; cultured 5 days at 28°C.

Medium	Composition of Medium (g./l.)	Yield (mg./ml.) (mean of 6 flasks)
1	C, 8·0; N, 0·6; K, 14·8; P, 0·2; Na, 200×10^{-3}; Cl, 300×10^{-3}; S, 66×10^{-3}; Mg, 50×10^{-3}; Ca, 7×10^{-3}; Fe, 2×10^{-3}; Zn, 450×10^{-6}; Mn, 250×10^{-6}; biotin, 10×10^{-6}	1·44
2	As 1 plus Co, 20×10^{-6}; Cu, 12×10^{-6}; Mo, 25×10^{-6}	1·56
3	As 2 plus I, 30×10^{-6}; B, 3×10^{-6}	1·60
4	As 3 plus nicotinamide, 500×10^{-6}; thiamin hydrochloride, 200×10^{-6}; *p*-aminobenzoic acid, 100×10^{-6}; riboflavin, choline chloride, sodium pantothenate, pyridine hydrochloride; all 50×10^{-6}	1·79
5	As 4 plus peptone, 5×10^{-3}	1·86
6	As 5 plus F, 45×10^{-6}; Br, 35×10^{-6}; Cd, 20×10^{-6}; Ni, 12×10^{-6}; Al, 4×10^{-6}	2·02
7	As 6 plus Hg, 40×10^{-6}; Ba, 25×10^{-6}; Cr, 10×10^{-6}	1·90

(*d*) *Esterase*

This enzyme was measured by its ability to decompose the diacetyl derivative of chlorphenol red (3×10^{-5}M) at pH 7.0 and 37°C.

(*e*) *Reducing Sugar*

The reducing sugar in the suitably diluted culture filtrate was estimated by the Nelson-Somogyi colorimetric method (Somogyi 1945). All substances present capable of reducing alkaline copper reagents would be estimated as "reducing sugar" by this technique. Since the apparent reducing sugar in the

medium usually fell to zero, error from this source could not have been very great.

(*f*) *Sucrose*

Sucrose was determined as in (*e*) after inversion with dilute hydrochloric acid.

TABLE 2

GROWTH OF *S. ATRA* IN A BASAL MEDIUM CONTAINING ACETIC ACID A.R. 15 ML., KNO_3 A.R. 1.0 G., $MgSO_4.7H_2O$ A.R. 0.5 G., KH_2PO_4 A.R. 3.0 G., KOH A.R. 16.5 G., AND D-BIOTIN 10 μG/L.

Medium adjusted to pH 7.0 (KOH) and trace elements removed according to the procedure of Steinberg (1950).

Additions						Yield of Mycelium (mg./ml.) After 5 Days at 28°C.
Fe^{++} (μg./l.)	Zn^{++} (μg./l.)	Mn^{++} (μg./l.)	Cu^{++} (μg./l.)	Mo (μg./l.)	Other Additions	
—	—	—	—	—	—	0·37
—	400	200	100	40	—	0·32
600	—	200	100	40	—	0·34
600	400	—	100	40	—	0·38
600	400	200	—	40	—	0·54
600	400	200	100	—	—	0·96
600	400	200	100	40	—	1·14
1200	400	200	100	40	—	1·30
600	800	200	100	40	—	2·28
600	400	400	100	40	—	1·66
600	400	200	200	40	—	1·26
600	400	200	100	80	—	0·60
600	400	200	100	40	I′ 300 μg./l., Br′ 250 μg./l., B 25 μg./l.	0·60
600	400	200	100	40	Plant ash (from wood of *Eucalyptus* sp.) 1 mg./l.	0·42
600	400	200	100	40	Mixed B vitamins at 10-100 μg./l.	1·96
600	400	200	100	40	Peptone 10 mg./l.	2·40
600	400	200	100	40	All the above additions	1·42

(*g*) *Total Carbohydrates*

The orcinol method of Pirie (1936) was suitable for the estimation of starch and its degradation products in S. *atra* culture filtrates.

(*h*) *Mycelial Weight*

The culture medium was filtered through a pair of matched ashless filter papers (Whatman No. 541), the mycelium washed once with distilled water, the whole dried 48 hr. at 55°C., and the mycelial weight obtained by difference.

IV. Mineral Nutrition of S. atra

The modified Waksman-Carey solution of Fahraeus (1947) is deficient in certain elements known to be important in the nutrition of fungi, including zinc, copper, and molybdenum (Steinberg 1950). When S. *atra* was grown in this medium with glucose or sucrose as a carbon source, addition of other elements did not affect the final yield of mycelium. The addition of $ZnSO_4.7H_2O$ (2 mg./l.) was found to eliminate the lag periods of many days between the germination of the spores and the onset of rapid growth, which occurred in an unpredictable way in the absence of zinc. Apart from this regulatory effect, ordinary "purified" sugars and A.R. chemicals apparently contained enough of the micronutrient elements for growth of the fungi.

Table 3

COMPARISON OF MINERAL NUTRITION OF *S. ATRA* WITH THAT OF *M. ECHINATA* (PERLMAN 1948)

Metal	*S. atra*, Present Work, Acetate Media		*M. echinata*, Perlman (1948), Glucose Media		
	Optimum Concentration (μg./l.)	Ratio of Mycelial Yield at Optimum to Mycelial Yield on Basic Medium	Optimum Concentration (μg./l.)	Concentration in Basic Medium (μg./l.)	Rate of Mycelial Yield at Optimum to Mycelial Yield on Basic Medium
Iron	600-1200	*c.*3	230-430	136	1·9
Zinc	>800	At least 6·5	*c.* 110	11	1·9
Manganese	>400	At least 4	*c.* 1000	3	2·3
Copper	100-200	*c.* 2·2	*c.* 300	1	1·3

When S. *atra* was grown on acetate media its dependence on various inorganic micronutrients became apparent. It was possible to show that the presence of all the mineral components of the Waksman-Carey medium except calcium was necessary for optimum growth when using unpurified reagents. Table 1 shows that certain further additions to the medium brought about stimulation of growth under the conditions used by a further 40 per cent. These observations were amplified by testing certain micronutrients in the more rigorous experiments set out in Table 2. Specially cleaned, silicone-treated glassware was used, the basal medium was purified by the calcium carbonate method of Steinberg (1950) and washed-spore suspensions were used for inoculation. Significant but not absolute dependence on iron, manganese, copper, and zinc was demonstrated, together with an apparently inhibitory effect of molybdenum in excess of 40 μg./l. Fair growth of the mould in "purified" basal medium apparently means that Steinberg's procedure is not completely effective in removing trace metals from acetate media. In Table 3 the results for the growth of S. *atra* are compared with those of Perlman (1948) for the growth of *M. echinata* in glucose media.

TABLE 4
UTILIZATION OF VARIOUS CARBON SOURCES BY *S. ATRA* GROWING ON A WAKSMAN-CAREY MINERAL MEDIUM
Concentration of carbon source, 1 per cent.; initial pH 7.0; grown 5 days at 28°C.

Carbon Source	Yield (mg./ml.)	Carbon Source	Yield (mg./ml.)
(*a*) Sugars		Cellulose: filter paper	++++§
D-Fructose	7·7	sawdust	+++§
Maltose	4·2	Sodium alginate	Nil
D-Galactose	4·1	Cellulose acetate	Nil
D-Glucose	3·2	(*e*) Miscellaneous sugar derivatives	
D-Mannose	2·1	Gluconic acid	3·0‡
D-Xylose	3·0	Galactonic acid	Nil
L-Rhamnose	2·9	Gulonic acid	Nil
Lactose	2·7	Ribonic acid	Nil
Sucrose	2·5	Saccharic acid	Nil
Cellobiose	2·3	Mucic acid	Nil
Trehalose	1·9	Fructose diphosphate	Nil
Raffinose	1·8	Cellobiose octa-acetate	+§
D-Lyxose	0·9	(*f*) Alcohols	
D-Arabinose	0·7	Methanol	Nil
D-Ribose	0·3	Ethanol	Nil
L-Arabinose	0·8*	*iso*-Propanol	Nil
D-Fucose	Nil	*iso*-Butanol	Nil
L-Xylose	Nil	*n*-Butanol	Nil
L-Sorbose	Nil	*tert*-Pentanol	Nil
D-Glucoseamine	Nil	*n*-Pentanol	Nil
(*b*) Sugar alcohols		*n*-Hexanol	Nil
Glycerol	5·3	Ethylene glycol	Nil
Mannitol	3·2	Propylene glycol	Nil
Arabitol	1·0	(*g*) Organic acids	
Dulcitol	0·9	Acetate	0·7
Adonitol	0·8†	Propionate	+¶
Inositol	0·3†	Butyrate	+
Sorbitol	Nil	*iso*-Butyrate	+
Erythritol	Nil	Valerate	+
(*c*) Glucosides		Oxalate	+
β-Methyl-glucoside	1·9	Malonate	+
α-Methyl-glucoside	1·3	Succinate	+
Salicin	1·3	Adipate	+
(*d*) Polysaccharides		Malate	0·1
Inulin	3·6	Lactate	+
Sodium carboxymethyl cellulose	2·5	Citrate	+
Starch	2·1	Benzoate	+
Dextrin	1·7	Salicylate	+
Pectin	2·5‡	Formate	Nil
Arabinic acid	1·3‡	Glycollate	Nil
Xylan	++++§	Tartrate	Nil
Chitin	++++§	Glycerophosphate	Nil
Cellulose: cotton wool	++++§	Pyruvate	Nil

* After 13 days incubation.
† After 10 days incubation.
‡ After 7 days incubation.
§ Residual insoluble substrate prevented mycelial weight determination.
¶ "+" indicates growth in non-weighable amounts.

TABLE 4 (*Continued*)

Carbon Source	Yield (mg./ml.)
(*h*) Organic acids; nitrate as N source	
Acetate	1·0
Malonate	0·5
Malate	0·4
Tartrate	0·1
Succinate	0·1
iso-Butyrate	+
Butyrate	+
Lactate	+
Citrate	+
Oxalate	+
Formate	Nil
Valerate	Nil

Mixed B vitamins and protein nitrogen exert an apparent stimulatory effect on the growth of *S. atra*; in view of the results of Marsh and Bollenbacher (1946) and Perlman (1948) this is unlikely to reflect the existence of absolute requirements.

V. Utilization of Carbon and Nitrogen Sources

(*a*) *Carbon Sources*

The substance to be tested was added to the basal medium at a final concentration of 1 per cent., and where necessary the pH of the medium was readjusted to 7.0. The results of tests using a number of substrates are set out in Table 4.

(*b*) *Nitrogen Sources*

The carbon source used was 1 per cent. glucose and a few experiments with acetate media are also reported. The medium was varied by adding K_2HPO_4 (3.3 g./l.) in place of $(NH_4)_2HPO_4$ and a nitrogen source equivalent to pH 7.0 The results are set out in Table 5.

(*c*) *Single Substrates as both Carbon and Nitrogen Sources*

A certain number of the amino acids and proteins were tested as sources of both carbon and nitrogen. The $(NH_4)_2HPO_4$ in the medium was again replaced by K_2HPO_4 and the nutrient was added in an amount sufficient to give 0.57 g. N/l. These results are set out in Table 6.

(*d*) *Adaptation to Various Media*

The results quoted in Tables 4, 5, and 6 are for direct transfer from growth on the potato-dextrose agar solid medium to growth in liquid culture. All media on which fair growth was not apparent by the fifth day were retained

TABLE 5

UTILIZATION OF VARIOUS NITROGEN SOURCES BY *S. ATRA* GROWING ON A WAKSMAN-CAREY MINERAL MEDIUM

Carbon source: 1 per cent. glucose; initial pH 7.0; incubated 5 days at 28°C.

Nitrogen Source	Yield (mg./ml.)	Nitrogen Source	Yield (mg./ml.)
(*a*) Inorganic		Potassium phthalimide	3·1
Potassium nitrate ..	6·4	Urethane	+
Potassium nitrite	4·0	Hippuric acid	Nil
Ammonium phosphate	3·2	(*e*) Purines	
Ammonium nitrate ..	2·9	Xanthine	4·0
Ammonium sulphate ..	2·7	Uric acid	3·1
Ammonium chloride ..	2·1	Caffeine	+
Ammonium carbonate ..	0·9	Theobromine	+
Potassium thiocyanate ..	+	(*f*) Growth factors	
Potassium cyanide ..	Nil	Choline chloride ..	2·3
Hydroxylamine		Nicotinamide	0·3
hydrochloride	Nil	Pyridoxine hydrochloride	0·3
Hydrazine sulphate ..	Nil	*p*-Aminobenzoic acid ..	Nil
(*b*) Amines		Thiamine chloride ..	Nil
Methylamine	Nil	(*g*) Amino acids	
Dimethylamine	1·5	Proline	5·7
Trimethylamine	1·2	Arginine	5·5
Tetramethylammonium		Glycine	4·9
bromide	+*	Valine	4·1
Ethylamine	1·7	Asparagine	3·4
n-Propylamine	0·4	Phenylalanine	3·1
iso-Butylamine	0·3	Leucine	2·2
n-Amylamine	Nil	Lysine	1·9
Ethanolamine	0·9	Isoleucine	1·8
Ethylenediamine	Nil	Histidine	1·5
Putrescine	0·2	Cystine	++++†
Cadaverine	+	Hydroxyproline ..	+
Pyridine	+	Aspartic acid	Nil
Piperidine	+	Glutamic acid	Nil
α-Amino pyrimidine ..	+	(*h*) Proteins	
Indole	Nil	Gelatin	3·3
(*c*) Urea and related substances		Peptone	2·6
Methyl urea	5·1	(*i*) Various N sources with	
Ethyl urea	4·9	acetate media	
Guanidine hydrochloride	4·5	Ammonium nitrate ..	4·6
Alloxan	4·2	Potassium nitrate ..	1·0
Allantoin	3·7	Ammonium phosphate	0·7
Urea	3·2	Urea	0·5
Methylisothiourea sulphate	1·7	Arginine	0·45
Nitroguanidine	1·2	Guanidine	0·4
Phenylurea	1·1	Proline	0·35
Creatine	0·8	Methylurea	0·35
Creatinine	0·5	Uric acid	0·3
Thiourea	+	Glycine + potassium nitrate	0·3
(*d*) Amides		Glycine	0·2
Acetamide	0·9	Potassium nitrite ..	0·2

* "+" Indicates growth in non-weighable amounts.

† Residual soluble substrate prevented mycelial weight determination.

on the shaking machine for 3 weeks; if no growth was apparent after this period the cultures were discarded. In a few cases, noted in the tables, scanty growth took place in this period.

TABLE 6

UTILIZATION OF VARIOUS SUBSTRATES AS CARBON AND NITROGEN SOURCES BY *S. ATRA* GROWING ON WAKSMAN-CAREY MEDIUM

Initial pH 7.0, incubated 5 days at 28°C.

Subtrate	Yield (mg./ml.)
(*a*) Amino acids	
Alanine	0·3
Valine	0·15
Proline	0·04
Arginine	0·03
Histidine	0·03
Glycine	+*
Lysine	+
Isoleucine	+
Asparagine	+
Phenylalanine ..	+
Cystine	Nil
Leucine	+
Hydroxyproline ..	+
(*b*) Proteins	
Haemoglobin ..	3·0
Gelatin	2·8
Tryptone	2·1
Zein	2·0
Egg albumen	1·9
Peptone	1·9
"Mould enzyme" ..	1·2
Casein	0·5
Ground wool	++++†

* "+" Indicates growth in non-weighable amounts.

† Residual insoluble matter prevented mycelial weight determination.

Mycelial transfers were made from sparse cultures to a fresh batch of medium and this process was continued so long as there were signs of adaptation to the new substrate. Serial transfers were also made from cultures on various media that had supported measurable amounts of growth in the original experiments. The results of these subcultures may be summarized as follows:

(i) Media supporting "good" growth (better than about 0.5 mg./ml. in 5 days) continued to support growth at least as well on continued subculture.

(ii) Some media that gave scanty growth ("+") supported luxuriant growth on continued subculture; in other cases the amount of visible growth decreased to zero after two or three subcultures. Thus adaptation was made to growth

on media with propionate and malate as carbon sources and on cyanide as a nitrogen source, but subculture proved impossible on media with succinate and lactate as carbon sources. It was eventually possible to transfer to the corresponding solid medium *S. atra* that had become adapted to growth in liquid media; the fungus has, for instance, been maintained for over a year on propionate — Waksman-Carey agar. The range of nitrogen sources that could be utilized by these adapted strains was much diminished, nitrate being strongly preferred.

(iii) The adaptation of *S. atra* to growth on media containing salicylate and benzoate was accompanied by visible "mutation" and after about 20 days of culture on the salicylate medium, *S. atra* began to show sparse growth. Subculture on salicylate medium led to profuse growth with the production of large quantities of insoluble yellow pigment. Transfer of the culture to agar slopes, whether of solidified salicylate medium or potato dextrose agar, gave olive colonies that showed no sign of reversion to the wild type.

Microscopically the cultures were characterized by globose spores borne on exceptionally long conidiophores. White, Yeager, and Shotts (1949) have shown that aggregates of *Stachybotrys* spores always contain a few globose spores of the *Memnoniella* type; their observations have been confirmed in this laboratory. When spores from successive subcultures on liquid salicylate media were examined, it was found that replacement of the ellipsoid *Stachybotrys* type by the globose *Memnoniella* type occurred over three transfers. Intermediate cultures showed both spore forms.

It would seem that *S. atra* is heterokaryotic, with salicylate acting as a selecting agent.*

VI. Growth and Enzyme Production on Various Media

(*a*) *General Observations*

The composition of the modified Waksman-Carey medium is such that growth of a micoorganism upon it with the consequent depletion of the ammonium nitrogen must lead to a sharp fall in pH. With *S. atra* the onset of growth is sudden and the consequent fall in pH extremely sharp; with no experimental technique so far devised has it been possible to synchronize this change in a large number of flasks.

The occurrence of the second smaller drop in pH during growth on sugar media is particularly erratic and no account of the factors controlling its appearance can yet be given. Discrepancies between the "chronological" and "physiological" ages of cultures vitiated all experiments in which flasks were removed from the shaker at intervals and the culture filtrates examined. The Woulffe-bottle method for following a single culture was therefore devised to overcome this difficulty.

It might appear that these troubles could be eliminated by buffering the medium; in Table 7 are set out the results of a series of experiments in which

* Morphological details of the new strain and a full account of the above and further experiments will be published elsewhere.

media were prepared containing increasing proportions of 1.0M $K_xH_{3-x}PO_4$, pH 7.0. The table shows that reproducible growth is not obtained even when the pH of the medium is held relatively steady.

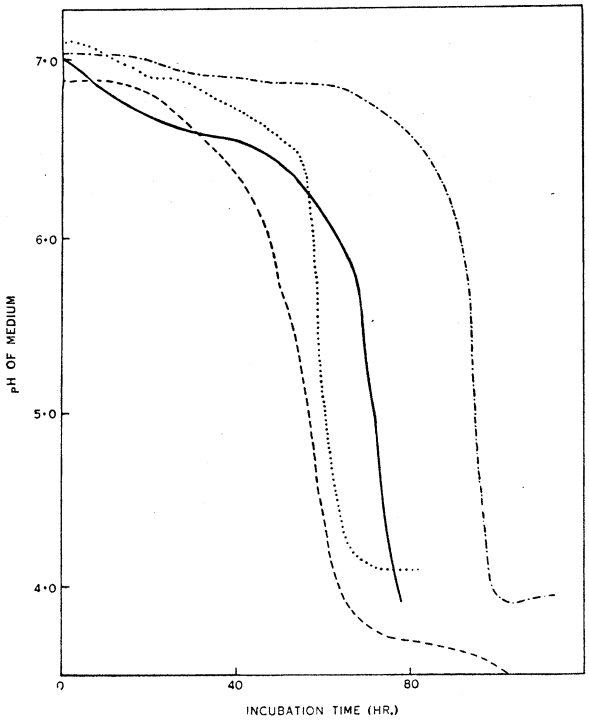

Fig. 1.—Changes in pH during the first 100 hr. of growth of S. *atra* on Waksman-Carey medium containing 2 per cent. of glucose. Four separate Woulffe-bottle cultures at 28°C.; pH followed on the recording pH meter.

The causes of the inherent variability in growth cannot at present be defined; in the experiments on the nutrition of the mould small amounts of vitamins or proteins added to the medium gave cultures with much reduced variability (cf. Table 1). It is possible that random variation in the carry-over of such stimulatory substances in the inoculum was responsible for the observed results.

Examination of culture filtrates by solvent extraction after the addition of excess mineral acid, followed by chromatographic investigation of the extract, showed the absence at all stages of organic acids in other than trace amounts. This is in agreement with the observations of Perlman (1948) on *M. echinata.* The production of organic acids by S. *atra* thus plays no part in the observed pH changes of the medium.

(*b*) *Changes in pH During Growth*

The variability of the time-pH curve for the first 100 hr. of growth on a medium containing 2 per cent. glucose is illustrated in Figure 1 for a number

TABLE 7

GROWTH OF *S. ATRA* ON WAKSMAN-CAREY MEDIUM CONTAINING VARIOUS CARBON SOURCES AND DILUTED WITH VARYING AMOUNTS OF 1.0M PHOSPHATE BUFFER OF pH 7.0

Individual 250-ml. flasks in shake culture at 28°C., containing 50 ml. of medium. "Enzyme activity" is the hydrolytic activity against sodium carboxymethyl cellulose

Volume per cent. of Phosphate Buffer in Final Medium	Carbon Source								Days Incubated
	Glucose (2%)			Sucrose (2%)			Cotton Wool (60 mesh: 2%)		
	pH of Medium	Enzyme Activity	Mycelial Weight (mg.)	pH of Medium	Enzyme Activity	Mycelial Weight (mg.)	pH of Medium	Enzyme Activity	
	3·60	1·46	103	2·20	0·49	231	6·58	0·11	3
	3·42	0·77	94	3·40	1·08	88	5·90	0·08	4
	3·48	0·83	90	3·55	0·99	94	5·60	0·28	5
	3·40	0·88	100	3·58	0·62	105	5·92	0·31	6
0	3·40	0·50	108	3·32	0·88	101	5·45	0·18	7
	3·38	0·87	98	3·30	1·00	115	5·30	0·28	10
	3·10	0·83	102	3·26	1·31	100	5·75	0·27	12
	3·42	1·28	100	2·20	0·12	379	5·12	0·23	14
	3·58	0·94	36	6·38	1·33	60	6·80	0·10	3
	3·70	0·29	135	4·30	0·51	244	6·75	0·05	4
	3·60	0·60	110	4·05	0·22	180	6·68	0·21	5
	3·85	0·62	61	6·12	0·09	173	6·62	0·24	6
5	3·62	0·52	63	5·92	0·03	236	6·52	0·27	7
	6·25	0·32	263	6·00	0·13	262	6·42	0·30	10
	3·78	0·53	100	6·30	0·04	225	6·50	0·40	12
	3·80	0·35	130	6·35	0·20	179	6·45	0·53	14
	6·00	1·05	114	6·55	1·22	51	6·90	0·14	3
	6·15	0·10	178	6·18	0·22	241	6·82	0·10	4
	6·05	0·27	161	5·82	0·03	258	6·82	0·32	5
	6·20	0·29	158	6·28	0·13	187	6·70	6·35	6
10	6·30	0·53		6·30	0·10	187	6·60	0·58	7
	6·50	0·14	130	6·15	0·02	215	6·70	0·70	10
	6·48	0·37	187	6·48	0·18	186	6·70	1·07	12
	6·52		110	6·28	0·02	264	6·50	0·69	14
	6·70	1·34	48	6·50	0·93	48	6·92	Nil	3
	6·52	0·18	184	6·10	0·13	204	6·98	Nil	4
	6·50	0·04	203	6·40	0·32	171	6·98	0·02	5
	6·75	0·40	186	6·52	0·25	187	6·52	0·35	6
20	6·52	0·10	133	6·65	0·13	163	6·80	0·52	7
	6·65	0·22	55	6·60	0·17	161	6·52	0·43	10
	6·80	0·38	299	6·80	0·08	176	6·52		12
	6·62	0·37	237	6·68	0·26	70	6·68	0·83	14

TABLE 7 (*Continued*)

Volume per cent. of Phosphate Buffer in Final Medium	Carbon Source								Days Incubated
	Glucose (2%)			Sucrose (2%)			Cotton Wool (60 mesh: 2%)		
	pH of Medium	Enzyme Activity	Mycelial Weight (mg.)	pH of Medium	Enzyme Activity	Mycelial Weight (mg.)	pH of Medium	Enzyme Activity	
35	6·80	1·22	30	6·92	0·50	19	7·00	0·10	3
	6·62	0·33	148	6·80	1·15	57	6·98	0·07	4
	6·50	0·10	108	6·70	1·53	35	6·95	0·28	5
	6·65	0·35	150	6·60	0·32	116	6·95	Nil	6
	6·50	0·34	124	6·62	Nil	221	6·80	0·14	7
	6·70	0·03	151	6·75	0·88	153	6·62	1·13	10
	6·80	0·04	87	6·92	0·06	251	6·90	0·17	12
	6·72	0·64	194	6·75	0·05	173	6·75	0·70	14
50	6·90	1·20	6	6·85	0·32	11	6·88	0·47	3
	6·88	1·35	10	6·95	1·43	21	7·00	0·25	4
	6·80	1·18	30	6·50	0·78	8	6·88	Nil	5
	6·55	0·24	141	6·75	0·37	30	6·90	Nil	6
	6·52	0·19	162	6·70	Nil	47	6·70	Nil	7
	6·52	0·04	115	6·72	0·14	5	6·82	1·06	10
	6·85	0·27	23	6·75	0·78	25	6·90	Nil	12
	6·70	0·28	165	6·70	0·09	40	6·75	0·27	14

of experiments. Figure 2 illustrates the complete course of the time-pH curves during typical growth on media containing 1, 3, and 5 per cent. glucose.

(*c*) *Enzyme Production on Various Media*

In Figures 3 and 4 are set out the essential details of growth on unmodified Waksman-Carey media with 2 per cent. glucose and sucrose, respectively, as the carbon sources. There is an overall similarity in the relation between C_x activity, mycelial weight, pH of medium, and utilization of sugar for cultures on the two media. Figure 4 shows the tendency, which has been general in these experiments, for salicinase to follow the same course as C_x and the *p*-nitrophenyl-β-glucosidase to pursue an independent course.

The data on which Figure 3 is based were secured before the importance of checking the activity of a number of enzymes was realized and no further continuous runs on this simple unmodified medium have been made. Later independent experiments have shown that the proteases (gravimetric and viscometric) are not demonstrable at any stage of growth; sporadic and extremely small activities can be demonstrated for esterase, amylase, and sucrase between the fifth and tenth days of growth.

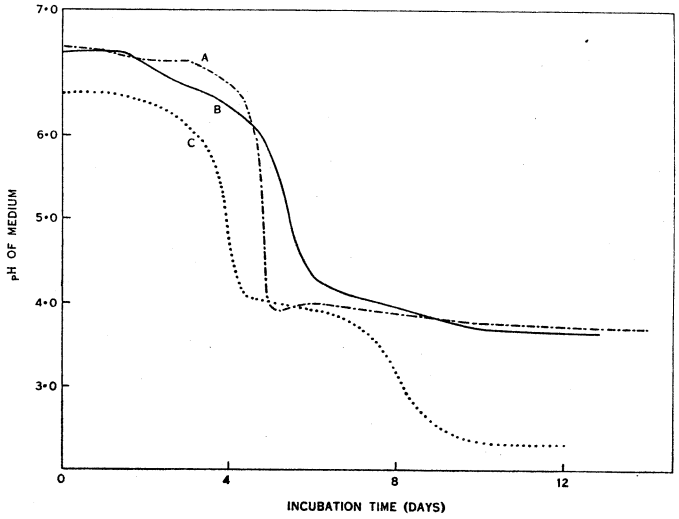

Fig. 2.—Changes in pH during the growth of *S. atra* on Waksman-Carey medium plus glucose. Woulffe-bottle cultures at 28°C.; pH followed on the recording pH meter. *A*, 1 per cent. glucose; *B*, 3 per cent. glucose; *C*, 5 per cent. glucose.

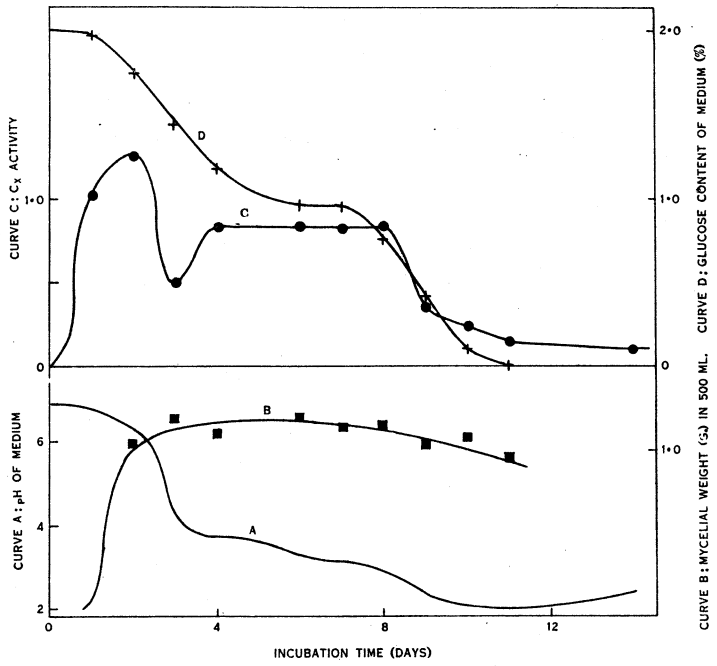

Fig. 3.—Growth of *S. atra* on 2 per cent. glucose medium. *A*, pH of medium; *B*, mycelial weight; *C*, "C_x" activity; *D*, sugar content of medium.

The growth of *S. atra* was also followed on glucose media modified in various ways; the results are set out in Figures 5, 6, and 7. It is apparent that each modification induces its own particular variation in the pattern of growth and enzyme production. In no case could the formation of significant amounts of amylase, sucrase, esterase, or protease be detected.

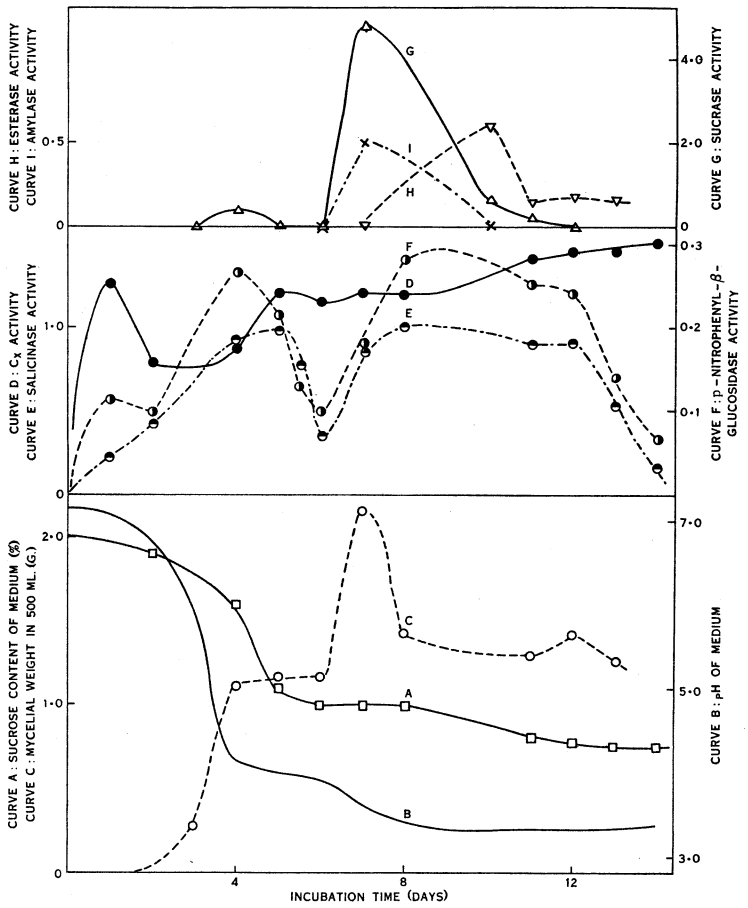

Fig. 4.—Growth of *S. atra* on 2 per cent. sucrose medium. *A*, sugar content of medium; *B*, pH of medium; *C*, mycelial weight; *D*, C_x activity; *E*, salicinase activity; *F*, *p*-nitrophenyl-β-glucosidase activity; *G*, sucrase activity; *H*, esterase activity; *I*, amylase activity.

When glucose is replaced by starch (Fig. 8), esterase, amylase, and sucrase are produced in some quantity and there is a general tendency for periods of maximum and minimum activity of these enzymes in the culture filtrate to coincide with those of the enzymes splitting β-glucosidic linkages. Of these latter enzymes C_x and salicinase do not exceed the maximum activities found in sugar media, but there is great increase in the *p*-nitrophenyl-β-glucosidase.

(*d*) *Comparison of Certain β-Glucosidase Activities in the Culture Media*

The comparative hydrolytic activity against salicin, *p*-nitrophenyl-β-glucoside, sodium carboxymethyl cellulose, and sodium cellulose sulphate was determined in bulked culture filtrates with the extra accuracy allowed by large sample size. The results are set out in Table 8.

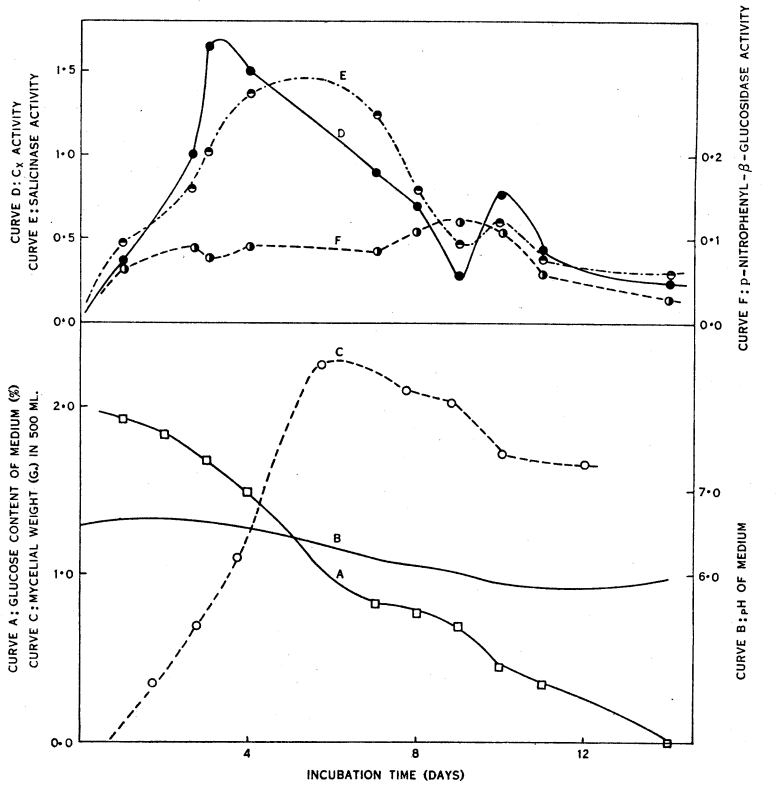

Fig. 5.—Growth of *S. atra* on 2 per cent. glucose medium plus 1/5 vol. of 1.0M phosphate buffer of pH 7.0. *A*, sugar content of medium; *B*, pH of medium; *C*, mycelial weight; *D*, C_x activity; *E*, salicinase activity; *F*, *p*-nitrophenyl-β-glucosidase activity.

VII. Discussion

There is no evidence of any unusual physiological characteristics in *S. atra*. The lists of available nitrogen and carbon sources supplement those of Perlman (1948) without any essential disagreement. The mould is not restricted to any type of nitrogen source, but as carbon sources shows a marked preference for sugars, polysaccharides, glucosides, and sugar alcohols, simpler molecules being utilized only in the presence of nitrate nitrogen.

TABLE 8

COMPARISON OF VARIOUS β-GLUCOSIDASE ACTIVITIES IN BULKED CULTURE FILTRATES

Carbon Source	Days of Growth	Hydrolytic Activity Against						
		p-Nitrophenyl-β-glucoside		Salicin		Sodium Cellulose Sulphate		Sodium Carboxymethyl Cellulose
		Arbitrary Units	SCMC-ase=1	Arbitrary Units	SCMC-ase=1	Arbitrary Units	SCMC-ase=1	Arbitrary Units
Glucose	2	9·0	0·11	64	0·75	86	1·0	85
	7	9·0	0·36	18	0·7	27	1·1	25
Sucrose	2	16·0	0·34	44	0·95	72	1·5	47
	5	8·7	0·14	37	0·65	47	0·75	64

Growth on unbuffered sugar media shows a definite sequence of phases:

(*a*) Germination, with considerable liberation of β-glucosidases before there is much apparent mycelial growth.

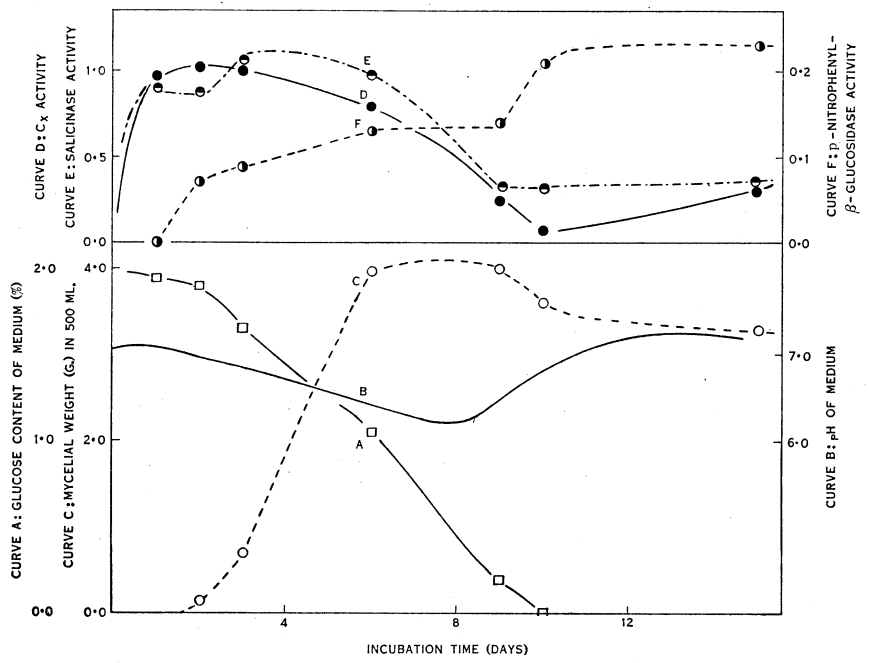

Fig. 6.—Growth of *S. atra* on 2 per cent. glucose medium plus 1 per cent. (w/v) of $CaCO_3$. *A*, sugar content of medium; *B*, pH of medium; *C*, mycelial weight; *D*, C_x activity; *E*, salicinase activity; *F*, *p*-nitrophenyl-β-glucosidase activity.

(*b*) Rapid growth, with fall in pH, consumption of sugars, and loss of C_x and salicinase activity.

(*c*) A steady period at pH 3-4, with a new rise in C_x and salicinase activity and little or no utilization of sugar.

(*d*) Renewed utilization of sugar, with a further slight decrease in pH and loss of C_x and salicinase activity.

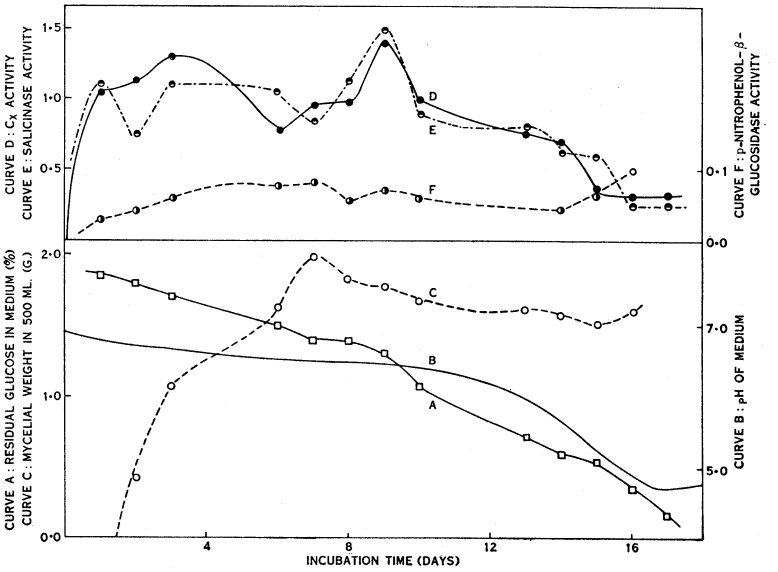

Fig. 7.—Growth of *S. atra* on 2 per cent. glucose medium modified by replacing $(NH_4)_2HPO_4$ with $K_2HPO_4 + KNO_3$. *A*, sugar content of medium; *B*, pH; *C*, mycelial weight; *D*, C_x activity; *E*, salicinase activity; *F*, *p*-nitrophenyl-β-glucosidase activity.

It is noteworthy that the first of these phases is not much altered by the addition of buffering agents or the replacement of ammonium by nitrate; secretion of relatively large amounts of β-glucosidases at, or just after, germination appears to be a constant feature of the metabolism of *S. atra*. Since the "C_x" activity is part of "cellulase" activity this early secretion of enzyme will be of the utmost importance for the mould in gaining a foothold on cellulosic substrates, and may be decisive in securing for *S. atra* the dominant position in the cellulolytic flora which it so often occupies in the field.

The secretion of "C_x" and salicinase appears to occur in bursts, with slow inactivation of the secreted enzyme thereafter; this is shown most clearly by the data for the two buffered glucose media, where the highest peak of activity occurs early in the history of the culture, and is followed by a nearly constant decline. On the limited evidence of the starch medium, this conclusion appears to be true for other enzymes also. The factor limiting growth in the media used was nitrogen; calculations show that approximately enough is present to synthesize about 1.6 g. of protein per 500 ml. of medium. Inspection of the

figures shows that except for the medium containing $CaCO_3$ growth ceased at a figure of about 2 g. of mycelium per 500 ml., followed by a drop in mycelial weight, and the liberation of enzymes. There were marked changes in pH, and the sugar continued to be utilized (presumably by respiration to CO_2). This partial autolysis could not be pushed further, however, and incubation for indefinite periods showed an almost constant mycelial weight. The residual mycelium was autolysed in all experiments (18 hr. with ethyl ether at 1°C.) but significant amounts of saccharases were not recovered.

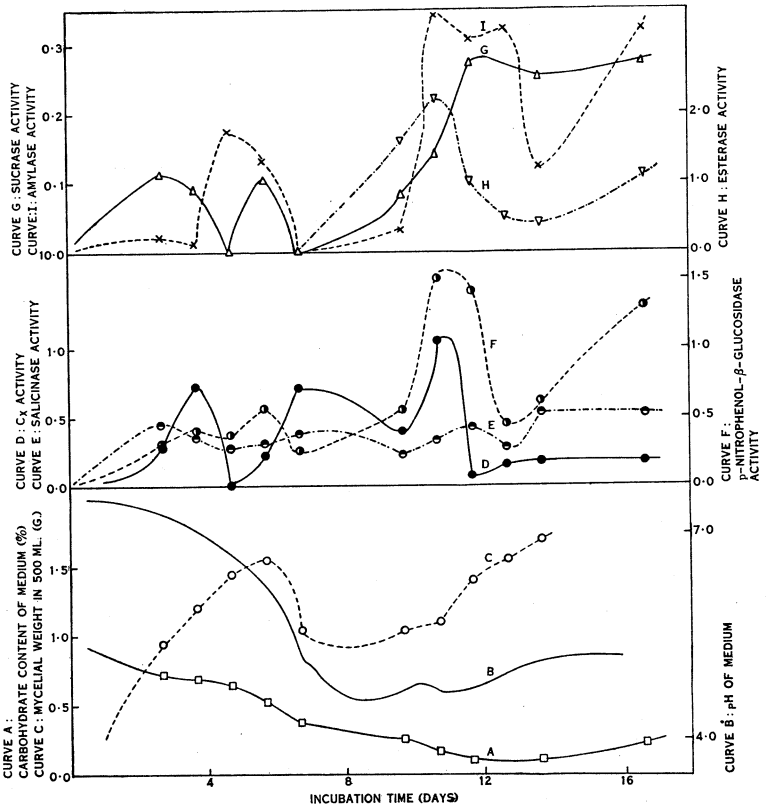

Fig. 8.—Growth of *S. atra* on 1 per cent. starch medium. *A*, carbohydrate content of medium; *B*, pH of medium; *C*, mycelial weight; *D*, C_x activity; *E*, salicinase activity; *F*, *p*-nitrophenyl-β-glucosidase activity; *G*, sucrase activity; *H*, esterase activity; *I*, amylase activity.

Shu and Blackwood (1951) have studied the production of certain carbohydrases by *A. niger* growing in submerged culture. Alpha-amylase and maltase activity were strongly dependent on the carbon source used, being higher on starch, dextrin, or maltose than on carbon sources not containing α-glucosidic linkages; "limit-dextrinase" activity on the other hand was almost independent of the carbon source. This situation is very similar to that found in this study for *S. atra*, amylase and sucrase production being dependent on the carbon source, and that of the C_x enzyme and other β-glucosidases being relatively

independent. Jermyn (1952*a*) has already shown that in general somewhat higher levels of the C_x enzyme are produced on media containing carbon sources with β-glucosidic linkages, so that this enzyme might be classified as "adaptive" if a few results only were considered. Contrary to the conclusions of Reese, Siu, and Levinson (1949) for cellulolytic moulds in general, however, its formation in large quantities by *S. atra* is possible in the absence of cellulose derivatives as carbon sources. The results obtained are apparently dependent on the balance of a number of factors, and this type of situation is fairly general; Gershenfeld and Wasserman (1950) found that, by choosing certain initial conditions, it was possible to demonstrate an apparently higher production of sucrase by *Neurospora crassa* growing on xylose, raffinose, and fructose than on sucrose, although a more complete study showed this enzyme to be partially adaptive.

The salicinase and C_x enzyme activities of *S. atra* follow courses that are sufficiently close to make it credible that the same enzymes are involved, although inspection of Figures 3-8 and of Table 8 shows that this relationship is not absolute; *p*-nitrophenyl-β-glucosidase appears to bear little or no relationship to the other two activities. The explanation advanced by Jermyn (1952*b*) for the properties of the *A. oryzae* β-glucosidases may therefore be applicable to those of *S. atra* and we may here have another example of a group of β-glucosidases each with its own ratio of activities towards different substrates, the sum of these activities being measured as the apparent activities of such "enzymes" as "salicinase" and "C_x." This hypothesis receives support from the tendency of salicinase and the C_x enzyme to have parallel maxima and minima of activity that are a day or two apart, as if a certain set of conditions was operating to produce or inactivate the β-glucosidases, but individual enzymes responded slightly differently.

The present study therefore gives no support for the existence of "C_x" as separate enzyme, hydrolysing polymeric β-glucosidic linkages only, and produced by the mould solely in response to the presence of these linkages.

VIII. Acknowledgments

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IX. References

Basu, S. N. (1948).—*J. Text. Inst.* **39**: T237.

Bisby, G. R. (1945).—*Trans. Brit. Mycol. Soc.* **28**: 11.

Buston, H. W., and Basu, S. N. (1948).—*J. Gen. Microbiol.* **2**: 162.

Cox, A. B., Hindson, W. R., Mortensen, K. N., and Wade, G. C. (1945).—Dep. Supply, Melb., Munitions Supply Lab. Rep. No. 170.

Crewther, W. G. (1952).—*Aust. J. Sci. Res.* B **5**: 290.

Crewther, W. G., and Lennox, F. G. (1953).—*Aust. J. Biol. Sci.* **6** (3) (in press).

Fahraeus, G. (1947).—*Symb. Bot. Upsaliens.* **9** (2).

Gershenfeld, L., and Wasserman, A. E. (1950).—*Amer. J. Pharm.* **122**: 146.

JENNISON, M. W., HENDERSON, R., DERRICK, M., O'NEILL, R., and RUPP, F. (1950).—Physiology of the wood-rotting fungi. Summary Tech. Rep. U.S. Off. Naval Res., Jan.-Dec. 1949.
JERMYN, M. A. (1952*a*).—*Aust. J. Sci. Res.* B **5**: 409.
JERMYN, M. A. (1952*b*).—*Aust. J. Sci. Res.* B **5**: 433.
LENNOX, F. G., and ELLIS, W. J. (1945).—*Biochem. J.* **39**: 465.
MARSH, P. B., and BOLLENBACHER, K. (1946).—*Amer. J. Bot.* **33**: 245.
PERLMAN, D. (1948).—*Amer. J. Bot.* **35**: 36.
PIRIE, N. W. (1936).—*Brit. J. Exp. Path.* **17**: 269.
REESE, E. T., SIU, R. G. H., and LEVINSON, H. S. (1949).—*J. Bact.* **59**: 485.
SAUNDERS, R. R., SIU, R. G. H., and GENEST, R. N. (1948).—*J. Biol. Chem.* **174**: 697.
SHU, P., and BLACKWOOD, H. C. (1951).—*Canad. J. Bot.* **29**: 113.
SIU, R. G. H. (1951).—"Microbial Decomposition of Cellulose." (Reinhold: New York.)
SIU, R. G. H., and SINDEN, J. W. (1951).—*Amer. J. Bot.* **38**: 284.
SOMOGYI, M. (1945).—*J. Biol. Chem.* **161**: 89.
STEINBERG, R. A. (1950).—*Arch. Biochem.* **28**: 111.
WAKSMAN, S. A., and CAREY, C. (1926).—*J. Bact.* **12**: 87.
WHITAKER, D. L. (1951).—*Nature* **168**: 1070.
WHITE, W. L., YEAGER, C. C., and SHOTTS, H. (1949).—*Farlowia* **3**: 399.