PRODUCTION OF ASPERGILLIC ACID BY SURFACE CULTURES OF ASPERGILLUS FLAVUS

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It has been reported by White (1940), Rake, McKee, and Jones (1942), White and Hill (1943), Jones, Rake, and Hamre (1943), and Bush, Dickison, Ward, and Avery (1945) that the fungus *Aspergillus flavus* produces an antibiotic substance known as aspergillic acid. *Aspergillus flavus* is also known to produce other antibiotic substances such as flavacidin (McKee, Rake, and Houck, 1943; McKee and MacPhillamy, 1943), and flavicin (Bush, Goth, and Dickison, 1945). However, for economical large-scale production of aspergillic acid, it was necessary to try additional media and methods of cultivation. The studies described in this report resulted in the development of suitable methods for obtaining valuable increases in the yields of material produced by the fungus.

METHODS OF CULTIVATION

White and Hill reported yields of 0.005 to 0.07 mg of crude crystalline material per ml of medium when *Aspergillus flavus* grew in surface culture at 23 C on a solution containing 2 per cent tryptone and 0.5 per cent sodium chloride. Rake *et al.* reported yields of 0.1 to 0.25 mg of crystalline aspergillic acid per ml of medium of the same composition. Bush *et al.* reported yields of 0.3 mg of crude crystalline material per ml of a solution containing 2 per cent Difco peptone and 2 per cent lactose. Similar results were obtained when the same media and methods were tried in this laboratory.

In the attempt to increase the yields in this laboratory several modified medium formulas were tried. Some were promising but others gave completely or nearly completely negative results. Individual media containing suitable sources of necessary nutrients such as soybean meal, vegetable meal, casamino acid, veal broth, Czapek-Dox, neopeptone, corn steep liquor, Brewer's yeast, and proteose peptone produced no detectable amount of aspergillic acid. A few other media which contained boiled potatoes, *dl*-isoleucine, Sabouraud's solution, or brain-heart infusion as the essential ingredient produced substantial amounts of aspergillic acid, but the one which gave the best yield as determined by assay was a simple solution containing 2 per cent Difco yeast extract and 1 per cent glycerol. This medium on the average yielded 0.8 mg of aspergillic acid per ml of solution in actual large-scale production lots and assayed over 1 mg per ml in the case of some smaller, experimental lots.

EXPERIMENTAL RESULTS

Fifty ml of the yeast extract glycerol medium were sterilized per 250-ml Erlenmeyer flask at 15 pounds for 15 minutes. The initial pH range was 6.3 to

6.6. The inoculum was 10^{10} spores in 1 ml of spore suspension. Incubation was at 25 C. These experiments represent several dozen flasks—each individual flask having been assayed biologically and spectrophotometrically.[1]

After inoculation of a flask, growth commenced promptly and by 48 hours a heavy, white, wrinkled pellicle was formed. The liquid under the pellicle was tested for activity and pH daily from the end of the third day until the twelfth day. The results of these experiments are shown in figures 1, 2, and 3.

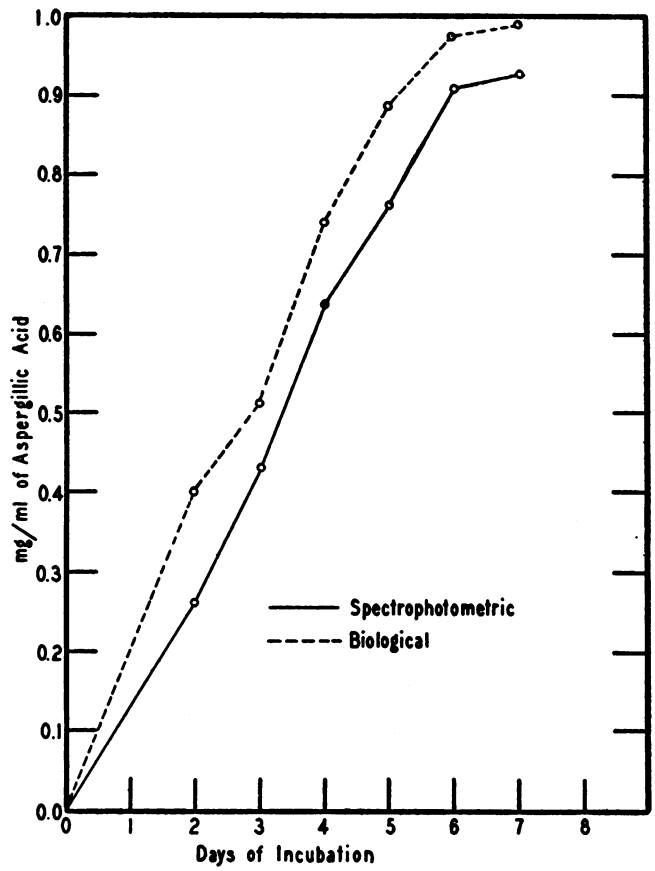

FIG. 1. ASPERGILLIC ACID—ASSAY VS. DAYS

It will be noted from the graphs that as the pH rises the activity also increases, and on the average the optimum incubation period is 6 to 7 days. The rise in pH is fairly rapid and consistent. The peak activity, as a rule, is obtained when the pH reaches 7.8. The greatest rise in pH occurs during the first two days as the pellicle is forming, and thereafter rises more slowly.

[1] Similar results were obtained in 110 production lots of 200 one-gallon bottles, each containing approximately 300 ml medium. As each production lot was harvested after 7 days' incubation, it was pooled and assayed as such both biologically and spectrophotometrically.

METHOD OF ASSAY

Previously, Rake *et al.* (1942, 1943) reported a rapid test for the activity of certain antibiotic substances, including aspergillic acid, based on the interference with the luminescence produced by luminescent bacteria. This interference can be directly correlated with antibacterial activity. However, in this laboratory two other methods were preferred: the spectrophotometric method which

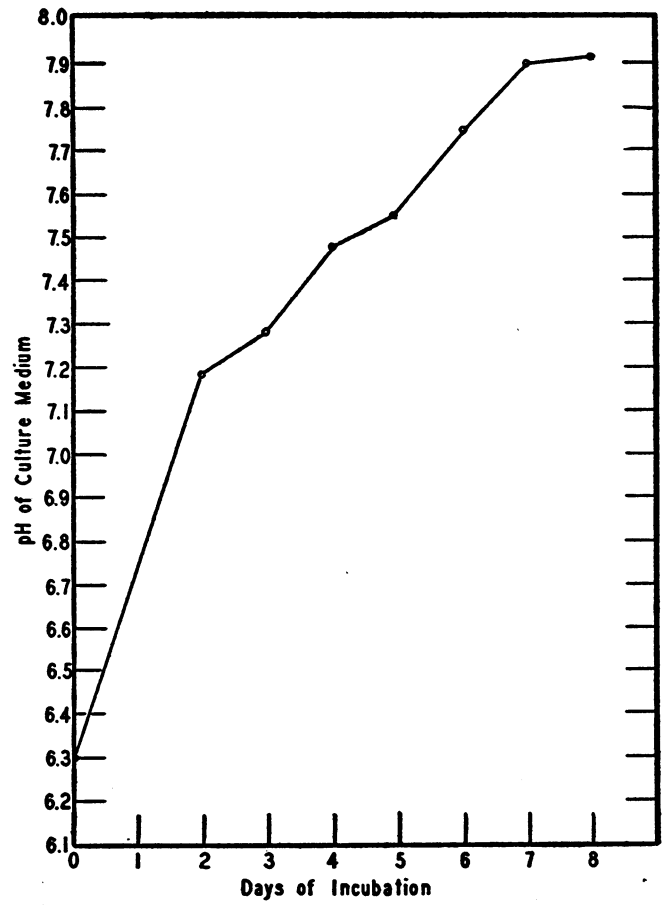

FIG. 2. ASPERGILLIC ACID, pH CURVE

is based on the ultraviolet absorption curve for aspergillic acid (maximum at 336 mμ in buffer), and the biological method in which activity is tested against a standard solution of aspergillic acid (serial tube dilution). The organism used in the latter test is the Heatley strain of *Staphylococcus aureus*.

The two assays confirm each other (Dr. J. D. Dutcher, to be published). Spectrophotometrically, it is impossible to differentiate between aspergillic acid and hydroxy-aspergillic acid. Biologically the two acids are completely dif-

ferent, the hydroxy-aspergillic acid being inactive, whereas the aspergillic acid is quite active. Physically and chemically the precipitated materials are not alike. Hydroxy-aspergillic acid has a melting point of 149 to 150 C and has 3 atoms of oxygen in its chemical structure, whereas aspergillic acid melts at 90 to 100 C and has but 2 atoms of oxygen in its chemical structure.

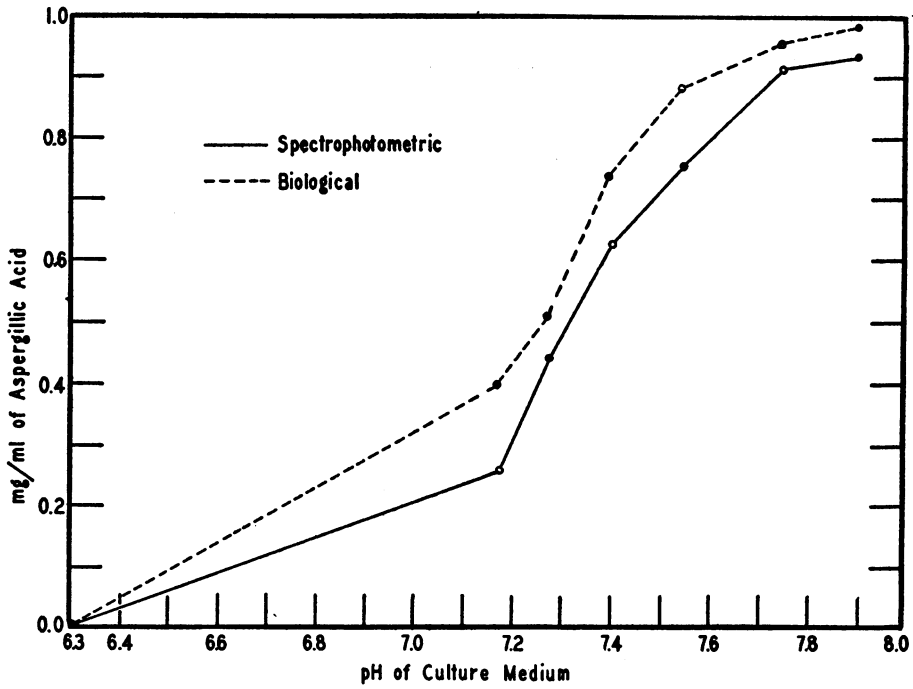

FIG. 3. ASPERGILLIC ACID—ASSAY VS. pH

TABLE 1

The effect of yeast on the yield of aspergillic acid

YEAST NO.	INITIAL pH	FINAL pH	SPECTRO-ASSAY	BIO-ASSAY
			mg/ml	*mg/ml*
333607	6.55	8.8	1.01	0.76
380588	6.5	8.9	0.03	0.68
378856	6.4	8.5	0.07	0.81
382370	6.5	8.75	0.85	0.60
382097	6.5	8.5	0.92	0.80

If a high titer in crude broth is obtained biologically, this must be verified spectrophotometrically, since *Aspergillus flavus* also produces penicillinlike substances. If a high titer in crude broth is obtained spectrophotometrically, this must be verified biologically to make certain that hydroxy-aspergillic acid is not being produced. If a high titer is obtained in both the chemical and bio-assay, a relatively large amount of aspergillic acid may be expected in the extraction or isolation process.

It is a relatively simple matter to produce hydroxy-aspergillic acid, but aspergillic acid is more difficult to produce. Experience has shown that not all lots of yeast extract will produce a high quantity of aspergillic acid.

Several lots of Difco yeast extract were tested and from the results in table 1 it can be seen that production of aspergillic acid depends on the yeast that is in the medium.

Yeasts 333607, 382370, and 382097 yielded the greatest amount of aspergillic acid, while yielding a small percentage of hydroxy-aspergillic acid. Yeasts 380588 and 378856 yielded practically no aspergillic acid or hydroxy-aspergillic acid, but did cause the formation of a penicillinlike substance.

SUMMARY

A method is described which enhances the production of aspergillic acid by *Aspergillus flavus* in surface cultures. A simple solution containing 2 per cent Difco yeast extract and 1 per cent glycerol yielded the highest titers of approximately 0.8 mg per ml in the crude broth. Emphasis is also placed on the importance of assay for aspergillic acid by both the spectrophotometric and the biological methods to verify the production of aspergillic acid, hydroxy-aspergillic acid, or penicillinlike substances.

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