determined by UV absorbance. Results are shown in Table I. The logarithm of $(C_0^1 - C_2)/C_0^1$ was plotted against time, and Fig. 6 was obtained. The slope of the straight line was calculated to be 0.0605 hr.$^{-1}$, and D_m/h to be 1.234 cm./hr. or 3.43 $\times$ 10^{-4} cm./sec. This same value was also used under *Appendix* of the previous report (1).

The Computation of Theoretical Values—The computations were executed by the help of the IBM 7090 digital computer. The simplified flow chart is shown in Fig. 7. Results are in Tables II and III and shown in Figs. 4 and 5.

Discussion—The excellent agreement of computed values and experimental results can be seen in Figs. 4 and 5.

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Keyphrases

Pyridine release from emulsions
Boundary effect on pyridine release as function of pH–w/o emulsions
Membrane effect on pyridine release–w/o emulsions
Diffusion equations, one-dimensional
Computer flow chart—theoretical values of pyridine release

Chemically Defined Medium for the Production of *Fusarium graminearum*

By ROBERT D. IMHOLTE and L. C. SCHRAMM*

A chemically defined medium for production of *Fusarium graminearum* has been developed and modified. A series of carbon and nitrogen sources was studied, resulting in the selection of glucose and ammonium succinate as primary substrates. Using the economic coefficient as a criterion for efficiency, the optimum concentrations for glucose and succinic acid were 3 and 0.5 percent, respectively. Growth weight and pH changes as well as glucose, nitrogen, and succinic acid utilization were determined during a typical fermentation period.

FUSARIUM GRAMINEARUM is the imperfect stage of *Gibberella zea* [*G. saubinetti* (Mont.) Sacc.], a plant pathogen causing stalk, root, and ear rot of corn and scab disease on wheat and barley. This organism has been classified in the order Hypocreales of the class ascomycetes. Ingestion of scabbed grains by domestic animals having simple stomachs elicits digestive system disturbances, vomiting, and in extreme cases, death (1–3). Bread made from scabbed grains has been called "intoxicating bread." Recent work on the fungus has resulted in the isolation of an anabolic uterotrophic compound (4, 5). Most investigators concerned with *F. graminearum* metabolites have utilized natural or incompletely defined substances as growth media. These have included potato infusion enriched with dextrose, scabbed barley, and cracked corn (1–5). The present report is concerned with the development of a completely defined chemical medium for the production of the fungus and a study of the growth habits on this medium. With such a medium it is possible to control the total environment of the fungus in order to facilitate extraction, metabolic, and animal toxicity studies.

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EXPERIMENTAL MATERIALS AND METHODS

A culture of *F. graminearum* was maintained by serial transfer on potato dextrose agar (PDA) slants. After initial growth for 1 week in an incubator at 28°, the cultures were stored at 4°. The liquid medium

used at the beginning of these experiments was derived from the PDA slant culture medium. It consisted of 20% potato infusion and 3% glucose, and was designated potato–dextrose broth (PDB).

Analytical determinations were accomplished in the following manner. A sufficient quantity of 500-ml. conical flasks, each containing 100 ml. of media, were plugged with disposable foam enclosures and autoclaved for 15 min. Inoculum was prepared by transferring a small portion of mycelium from a stock PDA slant to a flask of the same medium to be used for each experiment. This inoculum flask was incubated on a rotary shaker (1 in. diameter rotation, 300 r.p.m.) at 28° for 5–7 days. At the end of the incubation period, the contents of the flask were homogenized in a sterile Waring blender for 30 sec. The experimental flasks were inoculated by aseptically transferring a small loop of the well-stirred homogenized inoculum to each flask. The flasks were then incubated on the rotary shaker for a sufficient period of time. At arbitrarily specified intervals two flasks were harvested and treated as follows. The contents of each flask were filtered through a previously tared filter paper, the mycelium and filter paper frozen, then freeze-dried and weighed. The weight of the freeze-dried mycelium represented growth weight. The pH of the filtrate from the filtration described above was measured; a 15-ml. sample of the filtrate was preserved frozen. The quantity of glucose remaining in the filtrate was measured by the method of Shaffer and Somogyi (6). Nitrogen was measured by the method of Kala (7) and also by the Kjeldahl method.

RESULTS AND DISCUSSION

In an early investigation of the toxic metabolite of *F. graminearum* the PDB medium was devised to permit rapid production of large quantities of mycelium. When cultured in the PDB medium either in 500-ml. conical flasks on a shaker or in a 5-L. fermentor, the organism produced abundant growth and profuse quantities of red colored microconidia. Growth weight, pH, glucose, and nitrogen changes were determined during a typical incubation period of *F. graminearum* on this medium. The results are summarized in Fig. 1.

From this figure it can be ascertained that the maximum dry weight of mycelium obtained was

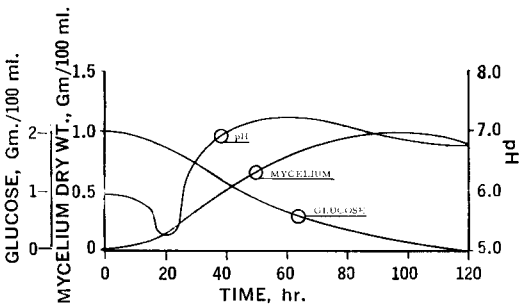

Fig. 1—Patterns of growth, pH changes, and glucose utilization of Fusarium graminearum *in potato–dextrose broth.*

0.94 Gm./100 ml., occurring approximately 100 hr. after inoculation. The pH of the medium varied from 5.25 to 7.25. The pH at the termination of the experiment (140 hr.) was 6.9. After an initial 15-hr. lag period, glucose was depleted in a logarithmic fashion during the logarithmic growth phase of the organism. (It is interesting to note that the minimum pH was recorded at the beginning of this logarithmic growth phase.) Glucose was apparently completely utilized at 110 hr. Maximum growth weight was reached before the glucose was depleted, indicating that glucose was not limiting growth. Nitrogen did not appear to be utilized since the slope of the nitrogen analysis regression line was −0.001. Because of the abundant production of the colored microconidia (about 8% of total dry weight) which hindered extraction studies, and because the mycelium produced in this medium was not acceptable to experimental animals as a food substitute in toxicity studies, it was necessary to investigate other media in anticipation that a completely defined synthetic medium might result from such further experimentation.

Semisynthetic media contain in part natural products and in part discrete chemical compounds. The absolute quantities of the chemical compounds may be varied between zero and the upper limits of solubility. Natural product constituents used in growth media are in many cases unknown. In addition, the reproducibility of the composition of a natural product is difficult due to the inherent variability of most biological systems. A synthetic medium would therefore be desirable if for no other reason than absolute control of all ingredients in the environment of the organism.

In order to evaluate and establish the limits of certain medium constituents capable of supporting growth, a number of semisynthetic media were constructed before attempting growth on a synthetic defined medium.

Glucose, mannitol, sucrose, and starch were screened singly and in combination, using neopeptone, yeast extract, and casein hydrolysate also singly and in combination as nitrogen sources. In these early screening experiments dry weight of mycelium after a 120-hr. incubation period was used as the criterion for efficiency. Although none of the media were as efficient as the original PDB medium, growth was supported by all media containing a nitrogen source; no growth was noted in media lacking nitrogen. Nitrogen was thus established as a requirement for growth.

Since *F. graminearum* did not appear to be a fastidious fungus, a chemically defined synthetic medium developed by Pacifici *et al.* (8) for the production of *Claviceps purpurea* was employed in the search for suitable substrates. This medium (see under *appendix*) not only supported growth, but was superior to PDB using terminal growth weight as criterion for efficiency.

The carbon and nitrogen modifications of Pacifici's medium are illustrated in Tables I and II.

Table I indicates that although Pacifici's original medium containing mannitol is capable of supporting growth of *G. zea*, either glucose or sucrose is more efficient in the combination with succinic acid. From the modifications made, it is apparent that a medium containing 3% glucose or sucrose and 0.1% to 1% succinic acid is most efficient. The criterion

TABLE I—CARBON SOURCE VARIATIONS—PACIFICI MEDIUM

Carbon Source, %	Succinic Acid, %	Mycelium Dry Wt.,[a] Gm.	Econ. Coeff.[b]
Mannitol, 3	3	1.59	63
Sucrose, 5	0.5	1.95	83.5
Sucrose, 3	3	1.23	48
Sucrose, 3	0.5	1.38	93
Sucrose, 1	0.5	0.56	87.5
Glucose, 5	3	2.03	61
Glucose, 3	3	1.72	68
Glucose, 3	1	1.65	101
Glucose, 3	0.5	1.50	105
Glucose, 3	0.1	1.29	104
Glucose, 3	0.05	0.84	69
Glucose, 1	3	0.85	49.5
Glucose, 0.5	3	0.47	31
...	3	0.45	34

[a] Average of three replicate flasks; 168 hr. fermentation. [b] Economic coefficient = mycelium dry wt./Gm. carbon consumed × 100.

TABLE II—NITROGEN SOURCE VARIATION—MODIFIED PACIFICI MEDIUM[a]

Nitrogen Source[b]	Mycelium Dry Wt.,[c] Gm.
Ammonium succinate[d]	1.72
Ammonium nitrate	0.46
Ammonium chloride	0
Ammonium formate	0
Ammonium bromide	0.18
Ammonium carbonate	0.32
Ammonium dihydrogen phosphate	0
Ammonium sulfate	0
Potassium nitrate	0.38
Calcium nitrate	0.78
Sodium nitrate	0.23
Neopeptone	0.20

[a] The basic medium consisted of 3% glucose, 3% succinic acid, and salts as listed in the Pacifici medium under *Appendix*. [b] Nitrogen sources were supplied in an amount equivalent to the nitrogen content of 3% ammonium succinate. [c] Average of three replicate flasks; 168 hr. fermentation. [d] Succinic acid was deleted from this modification.

for efficiency in this experiment was the economic coefficient; the greater numbers indicate greater utilization of carbon substrate. Succinic acid was considered as part of the carbon source in calculating the economic coefficient. For purposes of calculation, it was assumed that all sugar and succinic acid added to the medium was consumed.

The quantity of succinic acid does not appear to be critical between 0.1 and 1%. The economic coefficient drops sharply below 0.1% and above 1%. Using optimum succinic acid concentration, glucose appears to be the growth limiting factor at 3%; no further modifications of glucose concentration have been attempted.

Table II indicates that succinic acid, partially neutralized with ammonium hydroxide, is the most efficient nitrogen source. Although the nitrogen variations were accomplished before the optimum concentration of ammonium succinate was known, it is probable that similar results would be obtained using a nitrogen content equivalent to 0.1 to 1% ammonium succinate. Starting pH of the media was adjusted to 5.1–5.5 in all cases.

Variations in concentration of the major salts (magnesium sulfate and potassium hydrogen phosphate) indicated that the original quantities were in fact optimum.

No modifications of the micro elements have been attempted.

During the course of medium modification, a second analysis was accomplished. The medium used consisted of glucose 3%, succinic acid 3%, and salts as in Pacifici's original medium. Growth weight and pH changes as well as glucose utilization were determined. The results are summarized as Fig. 2.

Glucose was utilized in a logarithmic fashion, being depleted 80 hr. after inoculation. Growth weight did not reach a maximum until after glucose had been completely used, indicating utilization of succinic acid after glucose depletion. After an initial lag phase, the pH rose throughout the incubation period. The beginning of the rise coincided with the end of glucose utilization. The increase in pH could be explained on the basis of nonutilization of nitrogen (or only slight utilization) and metabolism of succinic acid after glucose had been almost completely depleted. This would account for the observed predominant ammonia odor in the medium, and the high ending pH (8.3). Maximum growth weight was 1.4 Gm./100 ml.

Decreasing the succinic acid to 5 Gm./L. (see under *Appendix*) resulted in still a different picture of metabolism. A fermentation similar to the previous ones was accomplished using the low succinic

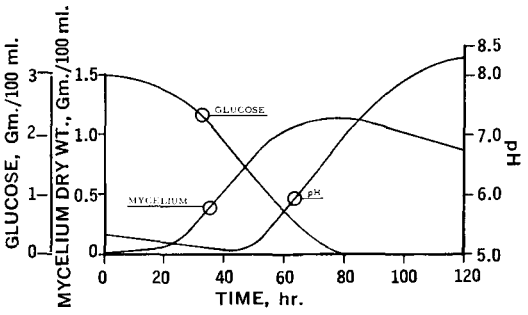

Fig. 2—Patterns of growth, pH changes, and glucose utilization of Fusarium graminearum *in the defined medium containing 3% succinic acid.*

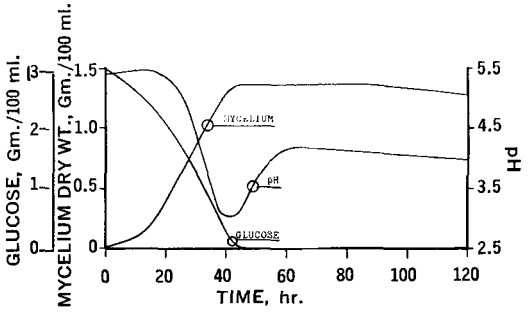

Fig. 3—Patterns of growth, pH changes, and glucose utilization of Fusarium graminearum *in the defined medium containing 0.5% succinic acid.*

acid concentration. The results are summarized in Fig. 3.

As in the previous experiment, glucose was utilized in a logarithmic fashion, being depleted in this case 45 hr. after inoculation. Growth weight, reached a maximum at the same time, indicating glucose as the growth weight limiting factor. After dropping from an initial pH of 5.4, the pH of the medium began to increase at the time glucose was depleted from the medium. It is probable that the succinic acid again was metabolized after glucose was depleted. In this fermentation, because of the low concentration of succinic acid, ammonia was also present in a much lower concentration. The final pH (4.0) was much lower than that encountered in other media. Maximum growth weight was 1.4 Gm.

SUMMARY

The experiments indicated above have resulted in the formulation of a completely defined medium for the production of *F. graminearum*. This medium was derived from one used for the production of another hypocrealic ascomycete, *Claviceps purpurea*. It appears that the optimal carbon sources are glucose (3%) and succinic acid (0.5%). Nitrogen is supplied in the form of ammonium hydroxide to adjust the pH of the medium to 5.1–5.5.

APPENDIX

Potato-Dextrose Medium (PDB)—Freshly diced potato, 400 Gm.; glucose, 30.0 Gm.; tap water *q. s. ad.* 1000 ml.

Pacifici Medium—Mannitol, 30 Gm.; succinic acid, 30 Gm.; KH_2PO_4, 1.0 Gm.; $MgSO_4 \cdot 7H_2O$, 0.3 Gm.; $FeSO_4 \cdot 7H_2O$, 14 mg.; $MnSO_4 \cdot 4H_2O$, 6.7 mg.; $ZnSO_4 \cdot 7H_2O$, 3.7 mg.; H_3BO_3, 2.5 mg.; KI, 0.76 mg.; $AlCl_3 \cdot 6H_2O$, 0.054 mg.; $CuSO_4 \cdot 5H_2O$, 0.03 mg.; NH_4OH *q.s.* to pH 5.5; distilled water *q. s. ad.* 1000 ml.

Defined Medium—Glucose, 30 Gm.; succinic acid, 5.0 Gm.; KH_2PO_4, 1.0 Gm.; $MgSO_4 \cdot 7H_2O$, 0.3 Gm.; $FeSO_4 \cdot 7H_2O$, 14 mg.; $MnSO_4 \cdot 7H_2O$, 6.7 mg.; $ZnSO_4 \cdot 7H_2O$, 3.7 mg.; H_3BO_3, 2.5 mg.; KI, 0.76 mg.; $AlCl_3 \cdot 6H_2O$, 0.054 mg.; $CuSO_4 \cdot 5H_2O$, 0.03 mg.; NH_4OH *q.s.* to pH 5.4; distilled water *q. s. ad.* 1000 ml.

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Keyphrases

Chemical media for *Fusarium graminearum*
Potato-dextrose broth
Glucose—succinic acid—ammonium hydroxide media
Glucose consumption—analysis
Nitrogen consumption—analysis