Production of yeast growth stimulants by molds on various media

H. H. Schopmeyer
Iowa State College

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UMI®
PRODUCTION OF YEAST GROWTH STIMULANTS BY MOLOS
ON VARIOUS MEDIA

BY

H. H. Schopfman

A Thesis submitted to the Graduate Faculty for the Degree of

DOCTOR OF PHILOSOPHY

Major subject Biophysical Chemistry

Approved

Signature was redacted for privacy.

In charge of Major work.

Signature was redacted for privacy.

Head of Major Department.

Signature was redacted for privacy.

Dean of Graduate College.

Iowa State College

1921
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ACKNOWLEDGMENT

The writer wishes to express his appreciation to Dr. Ellis I. Fulmer for suggesting the problem and for valuable advice and criticism from both Dr. Fulmer and Dr. L. M. Christensen during the progress of the work. Thanks are also due to Mr. I. R. Sipherd for aid in certain phases of the work.
HISTORICAL

Pasteur (1860) was one of the first to propagate yeast in nutrient solutions containing mineral salts and sugar. He used most successfully a medium consisting of 10 grams of sucrose, 0.1 gram of ammonium tartrate, one gram of yeast ash, and 100 c.c. of water, and inoculated this with a small bit of yeast the size of a pin-head. A small inoculation was followed by a slow fermentation which extended over a long period of time. A heavier inoculation gave a more rapid rate of evolution of gas bubbles, Pasteur's criterion for judging the rate of fermentation. In describing his experiments, he says in part: (translation)

"After 24 - 36 hours microscopic bubbles of carbon dioxide appeared, and on the following days they filled the flask and a deposit covered its bottom. Under the microscope a drop of this deposit revealed beautiful yeast-cells, much bunched and extremely young in looks, that is, the globules were turbid-translucent and non-granular; among these could be distinguished with surprising ease each globule of the small quantity of yeast used as seen; the young cells were infinitely more numerous than the old. It must not be thought that this solution fermentation ever became so active as it did when in place of using ammonia as the source of nitrogen, a suitable albuminoid substance was employed, such as that of grapes, beet juice, or yeast water. In sugar solutions containing one of these albuminoids fermentation was much more active; instead of after 24 - 36 hours, the first small bubbles appeared in 12 - 24, and much more yeast was formed in any given time. Nothing is more curious than this influence of the composition of the medium on the activity of the fermentation, and I have made a number of experiments on the matter. I was much surprised to find that in a medium formed by dissolving sugar in a filterate solution of albumin from the whites of eggs, yeast cells will not bud at all, and there is no trace of fermentation when blood-serum is used, or juices squeezed from muscles, the cells
develop with marvelous ease and the sugar ferments almost as well as in a natural saccharine juice or clear yeast-water; this is not because the albumen of eggs differs from that of serum but because blood contains other albuminoid constituents which by their special nature are suitable for the nourishment of yeast; for if the blood be coagulated, bled with water and then filtered to remove the coagulated albumen, sugar dissolved in the limpid filtrate forms a medium in which yeast multiplies and gives rise to well-characterized alcoholic fermentation; while if white-of-egg be treated in the same way, no fermentation at all occurs. These experiments were repeated many times, and always gave the same results. It is obvious that there may be very great differences between members of the group called albuminoids or proteins. I have also observed that certain proteins are much more favorable than others to the development of the lactic acid ferment; for example, the soluble part of gluten or casein, the nitrogenous residue of liquids in which alcoholic fermentation has taken place, etc.

The scientific questions with which we are concerned in this thesis one can promptly see originated with these experiments. It is clear that Pasteur recognized the importance of some "albuminoid materials" in the nutrition of yeast and other microbes. He went further, and questioned the possibility of cultivating yeast at all without them, and when his own experiments with sugar, yeast ash and amonium tartrate gave negative results and those of Baegoli the same, he promptly stated that Pasteur had deceived himself and what he took for yeast must be something else. However, he failed to follow Pasteur's suggestion to, "Come to Paris and see for yourself," (J. Pasteur, 1872) and apparently Pasteur had won his point.

Millier (1901) while working in Leu's laboratory at Angers, found that he could not raise a healthy crop of yeast in salts and sugar solutions unless a little yeast, yeast-water, peptone, or beef extract were
added. The unknown substance -- perhaps a whole category of substances -- which so improves the medium, he called provisionally "Bios", and thus the name is defined by him; also, he must be credited with the first attempts to prove the chemical identity of this newly discovered substance. Although he did not isolate and identify a substance of Bios properties, he did show that Bios is not contained in yeast ash, nor is it urea, alanine, tyrosine, asparagine, guanine, creatine, caustic, ovalbumin, or nucleic acid, for none of these substances can take the place of wort, yeast-water, peptone, or extract of beef.

Wildiers described his Bios as follows:

1. Soluble in water.
2. Insoluble in absolute alcohol and ether. 80% alcohol, however, allows a good extraction of Bios.
3. Hot present in yeast ash. Therefore, is not an inorganic substance.
4. Not destroyed by one-half hour boiling with 30% sulfuric acid.
5. It seems to be changed by 30 minutes boiling with 10% solution of sodium hydroxide.
6. Not precipitated by lead acetate.
7. Mixes readily through parchment.
8. Contained in Liebig's meat extract, commercial peptone, and beer wort.
9. Bios is not present in such substances as urea, asparagin, alanine, tyrosine, nuclein bases, adonine, guanine, thymus nucleic acid, creatine, peptic and
tryptic digestion products of albumin.

If Bios is necessary for yeast growth it is very difficult to explain Pasteur’s success and Liebig’s apparent failure to get yeast growth in synthetic media. Naturally, there have been many theories advanced to explain this very apparent discrepancy. Wildiers explained it on the basis that Pasteur used a large inoculum (a portion the size of a pin-head), while Liebig used a smaller bit of yeast. Wildiers contended that the Bios was introduced with the yeast in Pasteur’s case, while this was not true with Liebig, for he used a much smaller quantity of yeast.

These explanations have been very well summarized by Miller (1880):

“Liebig’s suggestion that Pasteur could not distinguish yeast from lactic acid microbes may be dismissed at once; the microscope was Pasteur’s favorite tool. Wildiers’ explanation that Pasteur added Bios with his yeast requires that Pasteur’s pins have heads of more than 0.1 c.c. in volume, for the drops of Wildiers’ own “strong yeast” were insufficient to cause healthy growth. That Liebig failed because he used too little yeast for seed can hardly be maintained; to him a pin-head meant 3 mm., he tells us so, and also that he used one-tenth of this amount —about a million cells— for seed. Wildiers’ suggestion that perhaps Liebig’s solutions were better supplied with air than were Pasteur’s and that this might have caused parts degeneration of the yeast as used for seed, is of course no better than a guess. Mesnier’s discovery, on the other hand, that bacteria in a yeast culture may generate Bios there, offers a plausible explanation of Pasteur’s famous crop; for it was only after 1850 that the technic of pure cultures was developed.

The explanation that to me seems likeliest, however, is founded on a difference long ignored: in those early days they spoke of “beer yeast,” implying as it were that “pigs is pigs;” the fact that Liebig used a Munich bottom-yeast seemed unimportant, at least no one for many years suggested that the race of yeast employed might make a difference.

Wildiers, who worked with “various commercial yeasts” and two race races, thought he had proved the need of Bios to be “a general characteristic of all beer yeasts;” and this conclusion was supported by the work, though not by the words, of Raschig. Sulzer, however, found a yeast in Fleischmann’s yeast cake which he kept alive and well
more than a year, by constantly transplanting in his Indian I made up of inorganic salts and sugar, although the crops were always less than those in west; to make quite sure that Bios was excluded, one sugar was replaced by another (a synthetic sugar) and the yeast flourished as before. This shows there is at least one yeast that gives fair crops in media free from Bios; might not Pasteur's have been another?

The subject of yeast growth stimulants known under the name of Bios has been adequately reviewed up to 1935 by Tanner (1935) and later developments by Buchanan and Fulmer (1950).

It was first shown by Fulmer and Nelson (1926) and by Fulmer, Nelson, and Laecker (1924) that Bios was of a multiplicative nature. They separated the water-soluble extracts of alfalfa into four fractions with alcohol and found that the optimum concentration of these fractions when used in pairs materially affected the total yeast count. This should not have been true if but one substance were present.

Miller and co-workers (1934) (Inoue, M. A. . . . 1934) have been able to fractionate the water-soluble extract of malt sprouts and from this water-soluble extract precipitate an inactive substance, Bios I, with barium hydroxide. The material not precipitated is called Bios II and is only slightly active. Great stimulation occurred when the two fractions were reunited in the proper proportions. Bios I was identified as an inositol (Bastott, . . . 1935). Bios II has been further fractionated by the use of acetic acid, acetic anhydride, or acetyl chloride (Sparkin, . . . 1928) into two fractions which can be activated with NaOH.
In an attempt to determine whether the organic compounds essential to yeast growth could be produced by other fungi, Koscioleks (Tanner, E. S. 1925) inoculated nutrient solutions simultaneously with small amounts of yeast and Penicillium glaucum. Yeast cultures inoculated with mold showed an active fermentation. This stimulation according to Kosciolek was not a mere symbiosis because dead mold mycelium would also give the same stimulation and growth, it was due to substances which were water-soluble.

It is contended by Suzuki and co-workers (1929) that yeasts are divided into three classes based on whether or not they need Bios:

1. Those that must have Bios for growth.
2. Those that are greatly accelerated by Bios.
3. Those that are not stimulated by Bios.

They state Saccharomyces cerevisae must have Bios for growth, which is in direct discord with the findings of Pulmer, Nelson, and White (1922) and considerable strain must be placed on Suzuki's theory in attempting a satisfactory explanation of Pulmer, Nelson, and White's work in which they grew yeast in a salt and synthetic methode medium. Suzuki does show, however, that Aspergillus oryzae when cultured on Bios free media does produce Bios in appreciable quantities.

It has been previously reported by Pulmer and Schoppeyer (1921) that during the growth of the molds Aspergillus niger, Aspergillus clavatus, or Trichoderma...
lignorum, on either glycerol or sucrose, there is produced in the medium a growth stimulant for yeast Saccharomyces cerevisiae. It is the object of this work to further study the production of the stimulant in the following respects:

1. To study various molds to determine whether or not the production of the stimulant is a common property of them.

2. To study the effect of various kinds and concentrations of media on the growth of molds and the production of stimulants.

3. To study the nature of the stimulant from some specific mold:
   a. The extra-cellular stimulant.
   b. The intra-cellular stimulant.

4. To compare the stimulant with other varieties of fungi, or fungi from other sources.
During the investigation of the growth of yeast in synthetic media, it has been observed that occasionally a greatly enhanced growth was shown in media which had been accidentally contaminated by molds. From this bit of information the idea was conceived that it might be possible that the mold was synthesizing or at any rate was liberating a substance or substances in the media which might act as a yeast growth stimulant. Therefore, a systematic study of this phenomenon was outlined to attempt to find out definitely whether or not the mold was actually producing a bios, and if in appreciable quantities, and also to find out if this was a general characteristic of molds, and also to study the bios produced by some particular mold.

Rather than to attempt to isolate at first the molds contaminating yeast lines and identify them, it was decided to start with pure cultures of these various fungi. The following molds were obtained:

1. *Aspergillus niger*
2. *Aspergillus clavatus*
3. *Trichoderma lignorum*
4. *Rhizopus nigricans* (plus and minus) (male and female)
5. *Penicillium roqueforti*.

Molds numbers 1, 2, 3, and 4 were furnished by Dr. Simson of the Botany Department while number 5 was obtained from Dr. Taylor of the Chemistry Department. (This culture
having previously been furnished by Mr. Gilman.)

In attempting this study two kinds of media were used, Medium 9 of Palmer, Nelson, and Sherwood (1941), which contained the following constituents per 100 cc:

- 0.186 gm. of MgCl₂
- 0.100 gm. of KH₂PO₄
- 10.00 gm. of sucrose

The pH of this medium as prepared was about 6.9. The other medium was a modification of Gaupeh according to Baylor, Neubrodt-Smith, and Collins (1930) with glycerine substituted for sucrose; its composition being as follows:

- MgSO₄ --- 0.5 gm.
- K₂HPO₄ --- 2.0 gm.
- KCl --- 0.5 gm.
- FeSO₄ --- 0.01 gm.
- NH₄Cl --- 1.3 gm.
- Glycerine --- 10.0 gm.
- Water to one liter.

The reasons for the glycerine were as follows:

1. To obtain an entirely different type of substrate;

2. To so far as possible eliminate anything of a directly biological origin;

3. To eliminate the possibility of the production of stimulative caramels through the high temperature sterilization that might result with sucrose. (Palmer and Nuescheman, 1927), Walser, M.H. Williams, A. L. and Yerman, C.H. (1931).
The procedure generally used was as follows:
The medium was prepared as indicated and placed in 500 c.c. quantities in two-liter Erlenmeyer flasks and sterilized at 15 pounds for twenty minutes and then cooled and inoculated from stock spores of the respective molds kept on agar slants in the ice box and set aside at room temperature, which remained rather constant at 20 degrees, and allowed to grow. The more rapidly growing molds had a luxuriant mycelium in two weeks or less while the slow growers seemed to be making so little progress that the time of two weeks was arbitrarily used for the period of mold growth. The mold pads were then removed and the remaining media were sterilized, part of them by steam and part by filtration. Before sterilizing, however, the glycerine medium was enriched by adding 100 grams of sucrose per liter, while the sucrose media were used as obtained.

For the steam sterilization the medium was placed in 50 c.c. quantities in 125 c.c. Erlenmeyer flasks and sterilized with steam at 15 pounds pressure for 30 minutes. The filtration sterilization was effected by filtering the material through a sterile Berkefeld filter, and then transferring 30 c.c. quantities into sterile 125 c.c. Erlenmeyer flasks. The flasks of media were then inoculated at the various indicated pH values (see Tables I and II) with a stock culture of Saccharomyces cerevisiae to a count of one. (Palmer, Nelson, and Sherwood, 1921). This culture was number 4226 of the American Type Culture Collection and
was carried in stock culture in Medium C. After inoculation
the flasks were incubated at 30 degrees centigrade for
48 hours and the yeast count taken at that time. The reason
for preparing the two series of flasks was to check the
heat stability of the suspected stimulant, and also to
avoid the excessive caramelization that would result from
heating the inverted sugar. The data for the 5 molds
both on glycerine and sucrose media are given in Tables I
and II. The data show quite definitely that there is a
stimulant produced by each of the several molds both on
glycerine and sucrose substrates. This material withstands
considerable heat since there is no appreciable decrease
in the stimulative properties of the media sterilized by
steam over that sterilized by filtration.

There is not a very great difference apparently
in the relative amounts of stimulants produced by the
various molds. Since the molds Aspergillus niger and
Aspergillus clavatus are the more rapid growers, they will
lend themselves most readily to further study.

It is interesting to note that the Penicillium
roqueforti has none of the toxic properties described by
Flaming (1929) as characteristic of certain of the
Penicilli. This point was checked with much care with
media from cultures of various ages. It was found in
one instance that a six weeks old culture had developed
a very decided toxicity toward the yeast when grown on
sucrose, but repeated attempts to check this point
failed.
### TABLE I

Counts Indicating Promotion of Growth by Various Folds

Grown on Sucrose Media

<table>
<thead>
<tr>
<th>Hold</th>
<th>Aspergillus niger</th>
<th>Aspergillus flavus</th>
<th>Trichoderma lignorum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Steam Filt.</td>
<td>Steam Filt.</td>
<td>Steam Filt.</td>
</tr>
<tr>
<td>pH of Media</td>
<td>3.2 6.2 3.2 6.3</td>
<td>2.7 6.3 3.7 6.3</td>
<td>5.5 6.3 5.5 6.3</td>
</tr>
<tr>
<td>Yeast</td>
<td>73 93 86 86</td>
<td>45 105 88 88</td>
<td>109 89 67 80</td>
</tr>
<tr>
<td>Count</td>
<td>81 101 91 97</td>
<td>48 115 89 88</td>
<td>105 93 90 83</td>
</tr>
<tr>
<td></td>
<td>75 111 87 88</td>
<td>47 113 86 93</td>
<td>103 85 78 92</td>
</tr>
<tr>
<td></td>
<td>71 107 85 93</td>
<td>53 136 85 93</td>
<td>93 86 83 92</td>
</tr>
<tr>
<td>Final pH</td>
<td>2.2 2.4 2.3 3.7</td>
<td>2.8 5.0 2.9 3.1</td>
<td>2.6 3.7 3.6 3.1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Hold</th>
<th>Penicillium roqueforti</th>
<th>Rhizopus nitrincicus (plus)</th>
<th>Rhizopus nitrincicus (minus)</th>
<th>Medium C for check</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Steam Filt.</td>
<td>Steam Filt.</td>
<td>Steam Filt.</td>
<td>Steam Filt.</td>
</tr>
<tr>
<td>pH of Media</td>
<td>4.0 6.9 4.0 6.9</td>
<td>2.7 6.9 2.7 6.9</td>
<td>2.7 6.3 2.7 6.3</td>
<td>6.3 6.9</td>
</tr>
<tr>
<td>Yeast</td>
<td>49 23 45 72</td>
<td>99 12 134 10</td>
<td>8 11 34 99</td>
<td>37 21</td>
</tr>
<tr>
<td>Count</td>
<td>23 22 50 72</td>
<td>34 12 126 7</td>
<td>5 10 37 75</td>
<td>26 22</td>
</tr>
<tr>
<td></td>
<td>98 15 133 8</td>
<td>3 8 103 77</td>
<td>30 23</td>
<td></td>
</tr>
<tr>
<td></td>
<td>31 84 93 71</td>
<td>185 13 156 2</td>
<td>3 3 99 80</td>
<td>33 20</td>
</tr>
<tr>
<td></td>
<td>95 18 119 12</td>
<td>5 7 110 62</td>
<td>24 18</td>
<td></td>
</tr>
<tr>
<td></td>
<td>80 14 137 6</td>
<td>3 9 92 73</td>
<td>23 22</td>
<td></td>
</tr>
<tr>
<td>Final pH</td>
<td>3.0 5.6 3.0 5.0</td>
<td>2.8 3.8 2.9 6.5</td>
<td>2.7 5.0 2.3 2.8</td>
<td>5.9 5.9</td>
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<tr>
<td>Hold</td>
<td>Aspergillus niger</td>
<td>Aspergillus clavatus</td>
<td>Trichoderma liasicum</td>
<td></td>
</tr>
<tr>
<td>-------</td>
<td>------------------</td>
<td>----------------------</td>
<td>---------------------</td>
<td></td>
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<tr>
<td></td>
<td>Steam Filt.</td>
<td>Steam Filt.</td>
<td>Steam Filt.</td>
<td></td>
</tr>
<tr>
<td>pH of Media</td>
<td>3.3 3.9 3.3 6.9 3.3 6.9</td>
<td>2.7 6.9 2.1 6.3 2.4 6.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yeast</td>
<td>8 55 7 63 50 24 45 74 6 131 2</td>
<td>3 51 5 73 46 88 58 83 4 116 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Count</td>
<td>3 54 11 63 53 93 49 77 7 116 4</td>
<td>8 51 5 73 46 88 58 83 4 116 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>7 49 5 63 43 97 52 72 5 133 7</td>
<td>7 53 6 57 53 103 47 69 3 103 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>6 53 8 65 45 59 53 89 3 137 4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Final pH</td>
<td>3.2 2.7 3.3 2.9 3.3 3.6 2.4 3.4 2.1 2.6 3.1 5</td>
<td></td>
<td></td>
<td></td>
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<table>
<thead>
<tr>
<th>Hold</th>
<th>Penicillium roqueforti</th>
<th>Rhizopus nigricans (plus)</th>
<th>Rhizopus nigricans (minus)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Steam Filt.</td>
<td>Steam Filt.</td>
<td>Steam Filt.</td>
</tr>
<tr>
<td>pH of Media</td>
<td>3.7 6.9 3.7 6.9 3.1 6.9 3.1 6.9</td>
<td>3.2 6.9 3.2 6.9 3.2 6.9</td>
<td></td>
</tr>
<tr>
<td>Yeast</td>
<td>34 54 35 35 72 79 77 73 74 56 78</td>
<td>33 48 36 30 73 65 76 63 84 61 59</td>
<td></td>
</tr>
<tr>
<td>Count</td>
<td>39 65 42 32 89 85 82 73 81 82 73</td>
<td>36 65 33 32 81 89 87 65 88 73 80</td>
<td></td>
</tr>
<tr>
<td></td>
<td>35 63 41 30 83 90 83 83 73 81 73</td>
<td>40 55 33 37 79 78 84 56 81 83 34</td>
<td></td>
</tr>
<tr>
<td>Final pH</td>
<td>3.4 2.7 3.4 5.1 2.3 3.5 2.3 3.0 2.3 2.4 2.4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
From the data in Tables I and II it is evident that a substance has been produced by the various molds which acts distinctly as a yeast growth stimulant. This material apparently is of an extra-cellular nature and seems to be quite stable to heat. It is shown quite definitely that it is not a product of the caramelization of the hydrolyzed sugars of the media by the fact that it is present in the glycerine as well as in the sucrose media.

However, since there had been much controversy in the sterilization of the sucrose media after the molds had grown and since, according to Fulmer and Huston (1937), this caramelization is capable of producing marked stimulation, the following experiments were carried out to prove that this was not a significant source of the stimulant. A 100 gram portion of sucrose was dissolved in about 400 c.c. of water and to this about 20 c.c. or 1 to 4 M HCl were added. The solution was allowed to stand for two days for inversion. It was then neutralized by adding NaOH to pH 5.9. The solution was made up to one liter, and one half of this sterilized by filtration, and one half by steam. A similar medium was made up using the same amount of HCl and NaOH, but adding the NaOH to neutralize the HCl before adding the sugar. This was sterilized in the same way. In addition to this Medium y was prepared and similarly sterilized. The data are given in Table III. These data show that there is a slight stimulation in the filtration sterilized inverted sugar, but not nearly enough stimulation to warrant saying that this was the reason for the enhanced growth when using the mold filtrates.
### Table III

**Studies on Invert Sugar**

<table>
<thead>
<tr>
<th>Method of Sterilization</th>
<th>Inverted Sugar</th>
<th>Sugar not Inverted</th>
<th>Medium C</th>
<th>Ear Check</th>
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<tbody>
<tr>
<td>Steam Filt</td>
<td>6.9</td>
<td>6.9</td>
<td>6.9</td>
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</tr>
<tr>
<td>Steam Filt</td>
<td>6.9</td>
<td>6.9</td>
<td>6.9</td>
<td>6.9</td>
</tr>
<tr>
<td>pH before Growth</td>
<td>4.9</td>
<td>4.6</td>
<td>4.9</td>
<td>4.6</td>
</tr>
<tr>
<td>Yeast Counts:</td>
<td>45</td>
<td>46</td>
<td>45</td>
<td>46</td>
</tr>
<tr>
<td></td>
<td>44</td>
<td>46</td>
<td>44</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>41</td>
<td>45</td>
<td>41</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td>47</td>
<td>47</td>
<td>47</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>46</td>
<td>42</td>
<td>46</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>40</td>
<td>45</td>
<td>41</td>
</tr>
<tr>
<td>Average Count</td>
<td>45</td>
<td>40</td>
<td>41</td>
<td>42</td>
</tr>
<tr>
<td>pH after Growth</td>
<td>3.8</td>
<td>3.8</td>
<td>4.6</td>
<td>3.8</td>
</tr>
</tbody>
</table>
In order to study the "Bios" content of molds as well as their "Bios" producing properties, it seemed desirable to attempt to get as rapid and luxuriant a growth of mycelium as possible and at the same time to attempt to increase the yield of products formed by them, acids, etc.

Since Aspergillus niger produced "Bios" in what appeared to be as large a quantity as any mold studied and seemed to be a mold that grew well and with considerable rapidity, it was chosen as the one to do further work on, and a medium highly suited for its growth was sought. Raulin (1863) was probably the first to substitute synthetic media for natural substrates, such as potato, carrots, yeast, and their extracts. He sought to replace the ash of yeast used by Pasteur with salts essential to growth, and through this formulated the well known Raulin solution. This medium had the following composition:

Water -- 1500 grams, cane sugar -- 70 grams, tartaric acid -- 4 grams, ammonium tartrate -- 4 grams, ammonium phosphate -- 0.6 gram, potassium carbonate -- 0.6 gram, magnesium carbonate -- 0.4 gram, ammonium sulfate -- 0.35 gram, zinc sulfate -- 0.07 gram, ferrous sulfate -- 0.07 gram, potassium silicate -- 0.07 gram.

Because this medium was so very complex, it has been considerably modified by various workers. Currie (1917) in his work on production of citric acid by Aspergillus niger proposed the following medium: Sucrose -- 125-150 grams, ammonium nitrate -- 2-2.5 grams, potassium dihydrogen phosphate -- 0.75 gram to 1 gram, magnesium sulfate -- 0.35 gram, hydrochloric acid to a pH to 3.4 to 3.5 in enough water to make a liter. Iron and zinc are not, he contends,
essential to maximum production of citric acid, but are essential
to the normal functioning of the fungi. This medium is to be
contrasted with the much more dilute medium originally used by
Gzapeck which was revised by Haylor, Smith, and Collins (1930) in
their work on *Penicillium roqueforti*. Duclaux (Jorgensen, (1925) )
developed the mold on Raunin's medium and he showed that oxalic acid
occurred as an intermediate product in the complete combustion of
sucrose to carbon dioxide. Welmer (Jorgensen, (1935) ) showed that
oxalic acid is sometimes absent during the growth of the fungus,
whereas, it is invariably present when potassium nitrate is used
as the source of nitrogen. If ammonium chloride is used no oxalic
acid is found.

Elfing (Jorgensen, (1935) ) found that there exist races of
*Aspergillus niger* which tend to generate citric acid under circum-
estances when otherwise oxalic acid is produced.

Pruess, Peterson, Steenbock, and Fred (1931) give the
following medium as one which produces very good yield of
*Aspergillus niger* mycelium: Ammonium nitrate — 10 grams, potassium
dihydrogen phosphate — 0.9 grams, magnesium sulfate — 5 grams,
ferric chloride — 0.16 gram, zinc sulfate — 0.05 gram, glucose —
40 grams, water — 1000 cc. With this medium the authors were able
to produce 25.8 grams of *Aspergillus niger* mycelium per 100 grams
of glucose.

Haylor, Smith, and Collins (1930) maintain that the source of
the nitrogen is a very important factor in the growth of molds
and give the following medium as producing the best growth of
(largest felts) *Pencillium roqueforti*: Magnesium sulfate — 0.5 gram,
dipotassium phosphate — 1.0 gram, potassium chloride — 0.5 gram,
ferrous sulfate — 0.01 gram, ammonium chloride — 5.3 grams, sucrose — 2.5 grams, enough water to make a liter of solution.

In the mold study to be carried out, it was desired to attempt to determine whether the molds produced a stimulant of an extra-cellular nature or an intra-cellular nature or both. Consequently, Medium 2 (Fulmer, Nelson, and Sherwood, 1921) was inoculated in 500 cc quantities with Aspergillus niger, the medium having been previously sterilized at 15 pounds pressure for 30 minutes in an autoclave. The growth of the mold was slow but after considerable time had elapsed (about 6 to 8 weeks) very good growth resulted. Addition of magnesium sulfate in half gram quantities per 500 cc of medium greatly speeded up the growth. The Aspergillus niger grew reasonably well in Baylor's medium and also in Baylor's medium modified by substituting 10 grams of glycerine for the 2.5 grams of sucrose. But Aspergillus niger refused to grow at all in medium containing only the following: Glycerine — 10 grams, ammonium chloride — 1.88 grams, di-potassium phosphate — 1.0 grams. This medium is essentially Fulmer, Nelson, and Sherwood's Medium 2 with glycerine substituted for sucrose. The reason for the failure to obtain growth in this medium might lie in the fact that the glycerine was of a high degree of purity, while the sucrose was of a commercial grade and consequently traces of mineral impurities might be in the sucrose which would not be present in the glycerine.

Herrick (1931) in his work on the production of kojic acid by Aspergillus flavus gives the following medium as the one he found to produce maximum yields of that compound: Magnesium
sulfate -- 0.5 gram, potassium chloride -- 0.1 gram, phosphoric acid -- 0.054 gram, ammonium nitrate -- 3.5 grams, dextrose -- 20 grams, water to a liter.

The previously discussed media were prepared in accordance with the directions of the authors and each was inoculated with a small quantity of spores of *Aspergillus niger*. The original source of this organism was unknown, it had been obtained from the Botany Department through the kindness of Dr. Gilman. The results of these experiments are given in Table IV.

**TABLE IV**

Data Comparing the Growth of *Aspergillus niger* on Various Media

<table>
<thead>
<tr>
<th>Worker</th>
<th>Time Grown</th>
<th>cc N/10 base per 10 cc of media</th>
<th>Wt. of mycelium per 500 cc of media</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fred, Peterson, Steenbock &amp; Pruess</td>
<td>14 days</td>
<td>11.7</td>
<td>6.17</td>
</tr>
<tr>
<td>Currie</td>
<td>14 days</td>
<td>22.4</td>
<td>6.18</td>
</tr>
<tr>
<td>Herrick</td>
<td>14 days</td>
<td>12.15</td>
<td>6.58</td>
</tr>
<tr>
<td>aylor, Smith, Collins, (modified Ozopck)</td>
<td>14 days</td>
<td>2.3</td>
<td>2.035</td>
</tr>
<tr>
<td>Tulnax, Nelson, &amp; Sherwood, Medium G</td>
<td>14 days</td>
<td>5.5</td>
<td>3.63</td>
</tr>
</tbody>
</table>

In obtaining these data the following procedure was carried out; to obtain the cc of tenth normal base equivalent to 10 cc of the media that had supported mold growth a 10 cc sample of
this material was diluted with distilled water, boiled to expel CO₂, and titrated to neutrality with tenth normal base in the presence of phenolphthalein. To obtain the weight of the mycelium the medium was filtered and the residue which consisted of mycelium washed with distilled water and dried at 60 degrees C.

Since these data are not conclusive, it was considered desirable to attempt to find as nearly as possible the optimum conditions of growth for *Aspergillus piper*. To do this, the following media were prepared:

1. Glycerine — 10 grams, K₂HPO₄ — 1 gram, NH₄Cl — 5.3 grams, water to one liter.
2. Glycerine — 10 grams, K₂HPO₄ — 1 gram, NH₄Cl — 5.3 grams, FeSO₄ — 0.01 gram, water to one liter.
3. Glycerine — 10 grams, K₂HPO₄ — 1 gram, NH₄Cl — 5.3 grams, MgSO₄ — 0.5 gram, water to one liter.
4. Glycerine — 10 grams, K₂HPO₄ — 1 gram, NH₄Cl — 5.3 grams, FeSO₄ — 0.05 gram, MgSO₄ — 0.5 gram, water to one liter.
5. Glycerine — 10 grams, K₂HPO₄ — 1 gram, NH₄Cl — 5.3 grams, FeSO₄ — 0.01 gram, MgSO₄ — 0.5 gram, water to one liter.
6. Sucrose — 10 grams, K₂HPO₄ — 1 gram, NH₄Cl — 5.3 grams, water to one liter.

These media were placed in 100 cc quantities in 500 cc Erlenmeyer flasks, sterilized with steam at 15 pounds for 15 to 30 minutes and inoculated with *Aspergillus piper* at the pH of the medium.
itself. The results are given in Table V.

### Table V

**Study of the Composition of the Media as Effecting Mold Growth**

<table>
<thead>
<tr>
<th>Media Number</th>
<th>Relative growth of sporeling</th>
<th>Sporulation</th>
<th>Wt. of mold per flask</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Very slight</td>
<td>None</td>
<td>0.035 grams</td>
</tr>
<tr>
<td>2</td>
<td>Very slight</td>
<td>None</td>
<td>0.040 grams</td>
</tr>
<tr>
<td>3</td>
<td>Fair</td>
<td>Much</td>
<td>0.27 grams</td>
</tr>
<tr>
<td>4</td>
<td>No growth</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>Fair</td>
<td>None</td>
<td>0.30 grams</td>
</tr>
<tr>
<td>6</td>
<td>Good</td>
<td>Much</td>
<td>0.45 grams</td>
</tr>
</tbody>
</table>

From the preceding experiment the following conclusions may be drawn for the concentrations used:

1. The presence of iron retards sporulation.
2. The presence of iron is not essential.
3. Magnesium is essential in glycerine media.
4. Magnesium is not essential in a medium containing commercial sucrose.

Much work has been done studying the effect of the reaction of the medium in its relation to mold growth. Cline (1919) contends that *Aspergillus niger* gives increasing percentage of germination as the acidity rises from pH 7.0 toward a maximum which is about 3.0. Currie (1917) claims that a pH of 3.4 to
3.5 is optimal for the growth of Aspergillus niger in a production of citric acid. Other workers have found that a growing range for this fungus varies from a pH of 3.3 to 3.9, showing that there is much less tolerance for alkaline than for acid conditions. To check this, Carrie's medium was prepared, placed in 500 cc quantities in two liter erlenmeyer flasks, sterilized with steam, inoculated with Aspergillus niger spores, hydrochloric acid being added to the medium in varying quantities to produce media of different pH. In addition to this, 0.01 gram of zinc sulfate was added to another. The mold was allowed to grow for fourteen days. At the end of that time, 10 cc samples of the solution under the mold were diluted with water, boiled to expel carbon dioxide and titrated with sodium hydroxide in the presence of phenolphthalein, and the mycelium from the various flasks was removed, washed by placing in a bunsen funnel and pouring water over it, then dried at 60 degrees C. and weighed. From the data one might conclude that there is even better growth at a pH of 3.0 than at 3.5 as recommended by Carrie. It was quite evident that sporulation was much retarded as the acidity increased. Further, the addition of small quantities of iron and zinc salts produced greatly enhanced growth with very little sporulation. The data for the preceding experiment are given in Table VII.
TABLE III

The Effect of pH on the Growth of Apprenillus micrur in Knauf's Medium

<table>
<thead>
<tr>
<th>pH</th>
<th>pH of Inoculum</th>
<th>% of Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.7</td>
<td>20.0</td>
<td>4.70</td>
</tr>
<tr>
<td>6.0</td>
<td>60.0</td>
<td>6.10</td>
</tr>
<tr>
<td>6.5</td>
<td>80.0</td>
<td>6.47</td>
</tr>
<tr>
<td>6.8</td>
<td>80.0</td>
<td>10.42</td>
</tr>
<tr>
<td>7.0</td>
<td>80.0</td>
<td>5.11</td>
</tr>
<tr>
<td>7.2</td>
<td>80.0</td>
<td>4.27</td>
</tr>
<tr>
<td>7.5</td>
<td>80.0</td>
<td>0.58</td>
</tr>
<tr>
<td>7.8</td>
<td>80.0</td>
<td>Very slight growth</td>
</tr>
<tr>
<td>8.0</td>
<td>80.0</td>
<td>no growth</td>
</tr>
</tbody>
</table>

Literature studies indicate that Apprenillus micrur is
decidedly ammonious with respect to its nitrogen requirements.
Giraud used sodium nitrate as a source of nitrogen in his
synthetic media. Mallard (1936) in studying the alkaline
decomposition by staphylococci, finds that the largest activity
from the addition of potassium nitrate than from the use of ammonium
chloride in the same quantity. Others contend that in
general ammonium oxalate tends to increase the production of
ammonium and that for soil growth, nitrogen is more readily
available in this form than in the nitrate form. In order
to study the effect of various sources of nitrogen, the
following media were devised. At the same time, it was deemed advisable to check the effect of iron salts in these various media. It was not considered necessary, however, to repeat the same experiment using zinc salts because of the similarity of the action of the two substances.

The media prepared were as follows:

1. Sucrose - 125 grams, ammonium nitrate - 3 grams, potassium dihydrogen phosphate - 0.75 grams, magnesium sulfate - 0.3 grams, water to one liter.

2. Same as number one but containing in addition 0.01 gram of ferrous sulfate per liter.

3. Sucrose - 125 grams, potassium nitrate 5.58 grams, potassium dihydrogen phosphate - 0.75 grams, magnesium sulfate - 0.3 grams, water to one liter.

4. Same as number three but containing in addition 0.01 gram of ferrous sulfate per liter.

5. Sucrose - 125 grams, ammonium chloride - 3.55 grams, potassium dihydrogen phosphate - 0.75 grams, magnesium sulfate - 0.3 grams, water to one liter.

6. Same as number five but containing in addition 0.01 gram of ferrous sulfate per liter.

The pH of each of these media was adjusted to 3.0 by adding hydrochloric acid after sterilization. The nitrogen present in each was in equimolar proportions. Five 500 cc Erlenmeyer flasks each containing 100 cc of the medium were run for each kind of medium. The data are given in Table VII.
From the data of Table VII it is evident that the ammonium ion as a source of nitrogen gives both a higher total titratable acidity and a larger quantity of mycelium per unit quantity of nutrient solution. In addition to this the iron
added tended to repress sporulation and caused a marked increase in mycelium.

It was now considered advisable to study the effect of both iron and zinc salts on the growth of *Aspergillus niger* over a rather wide range of concentrations. In order to do this a medium was prepared containing per liter:

- Sucrose - 135 grams, potassium dihydrogen phosphate - 0.75 gram, ammonium chloride - 3.55 grams, magnesium sulfate - 0.2 gram. 100 cc portions were placed in 300 cc Erlenmeyer flasks and the pH adjusted to 3.0 with hydrochloric acid after sterilization. To this medium zinc sulfate and ferrous sulfate were added in varying amounts from a stock solution.

In order to more accurately study the effect of these salts on the growth of the fungus, the acidity of the various flasks was titrated in the usual manner at intervals throughout a fourteen day growing period. The results obtained are given in Tables VIII and IX.

### Table VIII

**The Effect of Ferrous Sulfate and Zinc Sulfate On The Growth of *Aspergillus niger***

<table>
<thead>
<tr>
<th>Grams of ZnSO₄ per 100 cc</th>
<th>cc N/10 base for 10 cc</th>
<th>% of mycelium per 100 cc</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.005</td>
<td>11.4</td>
<td>4.37</td>
</tr>
<tr>
<td>0.01</td>
<td>12.2</td>
<td>4.34</td>
</tr>
<tr>
<td>0.05</td>
<td>14.55</td>
<td>3.92</td>
</tr>
<tr>
<td>0.1</td>
<td>18.15</td>
<td>Not reliable</td>
</tr>
<tr>
<td>0.5</td>
<td>23.5</td>
<td>because of</td>
</tr>
<tr>
<td>1.0</td>
<td>24.2</td>
<td>ZnSO₄ titration</td>
</tr>
<tr>
<td>2.0</td>
<td>20.3</td>
<td>0.11</td>
</tr>
</tbody>
</table>
### TABLE VIII (Continued)

<table>
<thead>
<tr>
<th>Grams of FeSO₄ per 100 cc</th>
<th>cc N/10 base for 10 cc</th>
<th>Wt. of mycelium per 100 cc</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.005</td>
<td>14.2</td>
<td>3.25</td>
</tr>
<tr>
<td>0.01</td>
<td>11.7</td>
<td>3.57</td>
</tr>
<tr>
<td>0.05</td>
<td>11.7</td>
<td>3.10</td>
</tr>
<tr>
<td>0.1</td>
<td>11.3</td>
<td>2.88</td>
</tr>
<tr>
<td>0.5</td>
<td></td>
<td>2.08</td>
</tr>
<tr>
<td>1.0</td>
<td></td>
<td>None</td>
</tr>
<tr>
<td>2.0</td>
<td></td>
<td>None</td>
</tr>
</tbody>
</table>

### TABLE IX

The Effect of Ferrous Sulfate and Zinc Sulfate on the Production of Acid and Mycelium by Aspergillus niger

<table>
<thead>
<tr>
<th>Grams of FeSO₄ per 100 cc</th>
<th>Grams of ZnSO₄ per 100 cc</th>
<th>cc N/10 base for 10 cc</th>
<th>Wt. of mycelium per 100 cc</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.005</td>
<td>0.005</td>
<td>13.5</td>
<td>3.47</td>
</tr>
<tr>
<td>0.01</td>
<td>0.01</td>
<td>15.8</td>
<td>3.56</td>
</tr>
<tr>
<td>0.05</td>
<td>0.05</td>
<td>11.7</td>
<td>3.80</td>
</tr>
<tr>
<td>0.1</td>
<td>0.1</td>
<td>9.6</td>
<td>3.97</td>
</tr>
<tr>
<td>0.5</td>
<td>0.5</td>
<td></td>
<td>2.96</td>
</tr>
<tr>
<td>1.0</td>
<td>1.0</td>
<td></td>
<td>0.15</td>
</tr>
<tr>
<td>2.0</td>
<td>2.0</td>
<td>No growth</td>
<td>0.0</td>
</tr>
<tr>
<td>Check 0</td>
<td>0</td>
<td>7.13</td>
<td>2.45</td>
</tr>
</tbody>
</table>

The experiment pertaining to ZnSO₄ and FeSO₄ was repeated using a narrow range of concentrations. In addition, the acidity was titrated at frequent intervals to determine the rate of its formation.
### TABLE X

Data Showing the Effect of FeSO₄ and of ZnSO₄ on Aspergillus niger over a Range of Concentrations

<table>
<thead>
<tr>
<th>Gms. of FeSO₄ per 100 cc</th>
<th>cc of N/10 base for 10 cc</th>
<th>Wt. of mycelium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3rd day</td>
<td>5th day</td>
</tr>
<tr>
<td>0.0001</td>
<td>3.6</td>
<td>3.22</td>
</tr>
<tr>
<td>0.0005</td>
<td>4.7</td>
<td>3.6</td>
</tr>
<tr>
<td>0.001</td>
<td>3.42</td>
<td>5.3</td>
</tr>
<tr>
<td>0.005</td>
<td>3.84</td>
<td>6.4</td>
</tr>
<tr>
<td>0.01</td>
<td>4.6</td>
<td>6.6</td>
</tr>
<tr>
<td>0.05</td>
<td>5.75</td>
<td>9.9</td>
</tr>
<tr>
<td>0.1</td>
<td>4.7</td>
<td>10.4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Gms. of ZnSO₄ per 100 cc</th>
<th>cc of N/10 base for 10 cc</th>
<th>Wt. of mycelium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3rd day</td>
<td>5th day</td>
</tr>
<tr>
<td>0.0001</td>
<td>5.4</td>
<td>10.7</td>
</tr>
<tr>
<td>0.0005</td>
<td>5.6</td>
<td>12.1</td>
</tr>
<tr>
<td>0.001</td>
<td>6.4</td>
<td>23.3</td>
</tr>
<tr>
<td>0.005</td>
<td>4.7</td>
<td>23.6</td>
</tr>
<tr>
<td>0.01</td>
<td>9.0</td>
<td>30.2</td>
</tr>
<tr>
<td>0.05</td>
<td>6.8</td>
<td>2.5</td>
</tr>
<tr>
<td>0.1</td>
<td>6.0</td>
<td>18.3</td>
</tr>
</tbody>
</table>

It is evident from the data in Tables VIII, IX, X, and XI that the addition of ZnSO₄ and FeSO₄ has a very marked effect on the production of both mycelium and acid. There is quite definite stimulation by FeSO₄ alone, much more stimulation with ZnSO₄ alone, and a very marked increase of stimulation by combinations of ZnSO₄ and FeSO₄. The optimum condition of these salts is not very definite but lies somewhere around 0.01 to 0.1 gram of each salt per liter of media.
The Effect of Mixtures of Ferrous Sulfate and
Zinc Sulfate on Production of Acid and Mycelium by Holts

<table>
<thead>
<tr>
<th>FeSO₄</th>
<th>ZnSO₄</th>
<th>Titratable acidity N/10</th>
<th>Wt. of mycelium per 100 cc</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>3rd day 5th day 7th day 10th</td>
<td></td>
</tr>
<tr>
<td>0.001</td>
<td>0.001</td>
<td>6.4 28.9 39.5 46.0 48.0</td>
<td>1.615</td>
</tr>
<tr>
<td>0.001</td>
<td>0.005</td>
<td>11.7 38.9 49.5 54.5 56.2</td>
<td>2.005</td>
</tr>
<tr>
<td>0.001</td>
<td>0.01</td>
<td>8.85 36.2 49.1 55.4 57.3</td>
<td>2.056</td>
</tr>
<tr>
<td>0.001</td>
<td>0.05</td>
<td>6.85 29.9 36.6 42.7 49.5</td>
<td>2.414</td>
</tr>
<tr>
<td>0.005</td>
<td>0.001</td>
<td>11.7 34.3 44.7 49.4 52.8</td>
<td>1.908</td>
</tr>
<tr>
<td>0.01</td>
<td>0.001</td>
<td>6.85 28.8 40.1 44.6 48.6</td>
<td>1.802</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>3.85 7.7 5.25 6.0 6.1</td>
<td>1.045</td>
</tr>
</tbody>
</table>

In a manner similar to that used in finding the optimum concentration of zinc and iron salts, magnesium sulfate was added to the following medium in varying amounts:
Sucrose - 135 grams, potassium dihydrogen phosphate - 0.75 gram, ferrous sulfate - 0.1 gram, zinc sulfate - 0.1 gram, ammonium chloride - 3.55 grams. This medium was placed in 500 cc quantities in two liter Erlenmeyer flasks and the acidity and weight of mycelium tested after two weeks growth. The acidity is distinctly low. The reason for this probably lying in the fact that with the larger quantity of medium the surface volume ratio was upset and was not so near the ideal surface volume ratio as one would obtain using 100 cc of media in a 300 cc flask. The data are given in Table XII. It will be noted that the optimum concentration of magnesium sulfate lies somewhere in the vicinity of 0.8 gram per liter, but as in the case of the zinc and iron salts this optimum is not extremely sharp.
### TABLE XII

**Effect of MgSO₄ on the Growth of Aspergillus niger**

**over a Range of Concentrations**

<table>
<thead>
<tr>
<th>Grams of MgSO₄ per 500 cc of media</th>
<th>cc of ⅕N base for 10 cc of media</th>
<th>Wt. of mycelium per 500 cc of medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>5.3</td>
<td>4.15</td>
</tr>
<tr>
<td>0.001</td>
<td>7.3</td>
<td>3.63</td>
</tr>
<tr>
<td>0.01</td>
<td>7.3</td>
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</tr>
<tr>
<td>0.1</td>
<td>16.8</td>
<td>15.03</td>
</tr>
<tr>
<td>0.2</td>
<td>14.9</td>
<td>14.13</td>
</tr>
<tr>
<td>0.4</td>
<td>15.4</td>
<td>14.23</td>
</tr>
<tr>
<td>0.8</td>
<td>14.9</td>
<td>14.100</td>
</tr>
<tr>
<td>1.6</td>
<td>14.9</td>
<td>14.03</td>
</tr>
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<td>2.0</td>
<td>19.2</td>
<td>14.95</td>
</tr>
<tr>
<td>3.0</td>
<td>18.2</td>
<td>13.02</td>
</tr>
<tr>
<td>4.0</td>
<td>15.8</td>
<td>13.11</td>
</tr>
</tbody>
</table>

Since nitrogen is said to exert a very marked effect on the growth of the fungus, the effect of varying the concentration of ammonium chloride was studied using the following medium: Sucrose - 135 grams, magnesium sulfate - 0.3 gram, ferrous sulfate - 0.1 gram, zinc sulfate - 0.1 gram, potassium dihydrogen phosphate - 0.75 gram. The above medium was placed in 100 cc quantities in 300 cc Erlenmeyer flasks, and the acidity titrated at intervals throughout the fourteen day growing period. It is extremely interesting to note that the production both of acids and of mycelium is very markedly affected by varying concentrations of ammonium chloride. It is also noted that the optimum concentration for the production of acids is quite distinct and lies around 1.875 grams of ammonium chloride per liter of solution. This is particularly interesting because this was the same optimum found by Fulmer,
Nelson, and Shervood, (1921) in their studies on the nutritional requirements of yeast. The optimum for production of mycelium is considerably higher than this lying around 3.5 to 3.5 grams per liter and is not nearly so sharply defined. The data for this experiment are in Table XIII.

### Table XIII

Data Showing the Effect of \( \text{NH}_4\text{Cl} \) over a Range of Concentrations

<table>
<thead>
<tr>
<th>Grams ( \text{NH}_4\text{Cl} ) per 100 cc</th>
<th>3rd day</th>
<th>5th day</th>
<th>7th day</th>
<th>9th day</th>
<th>11th day</th>
<th>14th day</th>
<th>per 100 cc</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.07</td>
<td>1.60</td>
<td>1.55</td>
<td>1.60</td>
<td>1.60</td>
<td>1.60</td>
<td>0.603</td>
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<tr>
<td>0.005</td>
<td>1.32</td>
<td>1.42</td>
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<td>3.62</td>
<td>3.62</td>
<td>2.173</td>
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<tr>
<td>0.05</td>
<td>5.54</td>
<td>5.05</td>
<td>5.45</td>
<td>5.40</td>
<td>5.50</td>
<td>5.50</td>
<td>5.885</td>
</tr>
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<td>0.1</td>
<td>14.95</td>
<td>16.00</td>
<td>17.50</td>
<td>17.75</td>
<td>18.25</td>
<td>18.25</td>
<td>1.273</td>
</tr>
<tr>
<td>0.125</td>
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<td>20.50</td>
<td>21.70</td>
<td>22.40</td>
<td>23.55</td>
<td>23.65</td>
<td>1.633</td>
</tr>
<tr>
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<td>33.22</td>
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<td>33.35</td>
<td>37.00</td>
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<td>40.05</td>
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<td>43.00</td>
<td>1.955</td>
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<td>37.90</td>
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<td>38.00</td>
<td>39.60</td>
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<tr>
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<td>38.30</td>
<td>46.90</td>
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<td>55.00</td>
<td>56.30</td>
<td>56.30</td>
<td>5.135</td>
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<tr>
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<td>74.50</td>
<td>75.50</td>
<td>67.85</td>
<td>64.33</td>
<td>2.665</td>
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<td>51.10</td>
<td>73.35</td>
<td>74.40</td>
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<td>63.20</td>
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<td>45.00</td>
<td>18.65</td>
<td>5.53</td>
<td>2.915</td>
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<tr>
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<td>38.07</td>
<td>9.00</td>
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<td>46.70</td>
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<td>3.095</td>
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<td>47.30</td>
<td>30.80</td>
<td>7.35</td>
<td>5.65</td>
<td>3.375</td>
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<td>13.86</td>
<td>35.20</td>
<td>35.63</td>
<td>20.81</td>
<td>7.45</td>
<td>6.83</td>
<td>2.665</td>
</tr>
<tr>
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<td>13.45</td>
<td>30.40</td>
<td>21.26</td>
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<td>7.05</td>
<td>7.05</td>
<td>3.445</td>
</tr>
<tr>
<td>0.350</td>
<td>12.85</td>
<td>29.30</td>
<td>28.00</td>
<td>8.50</td>
<td>6.60</td>
<td>7.30</td>
<td>2.445</td>
</tr>
<tr>
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<td>9.25</td>
<td>7.25</td>
<td>7.67</td>
<td>2.66</td>
</tr>
<tr>
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<td>11.73</td>
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<td>11.30</td>
<td>10.51</td>
<td>2.44</td>
</tr>
<tr>
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<td>13.24</td>
<td>16.45</td>
<td>17.75</td>
<td>17.20</td>
<td>18.40</td>
<td>2.49</td>
</tr>
<tr>
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<td>11.73</td>
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<td>17.32</td>
<td>20.00</td>
<td>19.10</td>
<td>19.33</td>
<td>2.33</td>
</tr>
</tbody>
</table>

In a similar manner the optimum concentration of potassium dihydrogen phosphate was found using the following
medium: Sucrose - 1.85 grams, ferrous sulfate - 0.1 gram, zinc sulfate - 0.1 gram, ammonium chloride - 1.875 mg., magnesium sulfate - 0.8 gram, per liter of solution, potassium dihydrogen phosphate being added in varying amounts from a stock solution. (The procedure in varying the concentrations of these various salts was exactly the same. The medium was prepared in such a concentration that 90 cc contained nutrients equivalent to 100 cc. This allowed 10 cc for dilution. The required amount of salt solution being studied was added, then sufficient distilled water added to make up the total volume to 100 cc per flask.) In the case of potassium dihydrogen phosphate series of flasks, the pH was not adjusted but was left that of the medium. This was to evade the difficulty arising from the buffer action of the potassium dihydrogen phosphate. The optimum concentration for the potassium dihydrogen phosphate, it will be noted, lies around 1.25 grams per liter and is rather sharply defined. This optimum holds rather closely both for acid production and for production of mycelium. The data are given in Table XIV.

In an effort to determine the optimum concentration of sucrose the following medium was devised: Potassium dihydrogen phosphate - 1.85 grams, ferrous sulfate - 0.1 gram, zinc sulfate - 0.1 gram, magnesium sulfate - 0.8 gram, ammonium chloride - 1.875 grams. Sugar was added to this in varying amounts and the acidity adjusted to a pH of 3.0
with the aid of hydrochloric acid. Fermentation was carried out in 100 cc quantities of medium in 300 cc Roux-meyer flasks. The yield of acid was extremely low as compared to previous yields and gave the maximum yields in concentrations of 18% to 19% sucrose. The data are given in Table XV.

### Table XV

<table>
<thead>
<tr>
<th>Grams</th>
<th>(KH_2PO_4)</th>
<th>cc of N/10 base for 10 cc</th>
<th>Weight of mycelium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pe$$\times$$100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cc of media</td>
<td>4th day</td>
<td>6th day</td>
<td>8th day</td>
</tr>
<tr>
<td>0.001</td>
<td>0.86</td>
<td>1.71</td>
<td>3.8</td>
</tr>
<tr>
<td>0.005</td>
<td>1.49</td>
<td>3.00</td>
<td>3.4</td>
</tr>
<tr>
<td>0.01</td>
<td>2.35</td>
<td>4.7</td>
<td>6.4</td>
</tr>
<tr>
<td>0.03</td>
<td>10.3</td>
<td>17.1</td>
<td>19.6</td>
</tr>
<tr>
<td>0.05</td>
<td>14.5</td>
<td>33.5</td>
<td>33.6</td>
</tr>
<tr>
<td>0.07</td>
<td>15.3</td>
<td>35.9</td>
<td>41.2</td>
</tr>
<tr>
<td>0.09</td>
<td>21.1</td>
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<tr>
<td>0.09</td>
<td>35.4</td>
<td>48.8</td>
<td>59.3</td>
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<td>28.6</td>
<td>58.1</td>
<td>60.2</td>
</tr>
<tr>
<td>0.135</td>
<td>22.9</td>
<td>50.0</td>
<td>70.1</td>
</tr>
<tr>
<td>0.150</td>
<td>22.6</td>
<td>47.0</td>
<td>56.0</td>
</tr>
<tr>
<td>0.175</td>
<td>24.3</td>
<td>47.0</td>
<td>53.6</td>
</tr>
<tr>
<td>0.300</td>
<td>31.8</td>
<td>45.6</td>
<td>42.8</td>
</tr>
<tr>
<td>0.350</td>
<td>34.3</td>
<td>39.9</td>
<td>42.6</td>
</tr>
<tr>
<td>0.300</td>
<td>27.8</td>
<td>39.8</td>
<td>40.1</td>
</tr>
<tr>
<td>0.40</td>
<td>33.5</td>
<td>34.2</td>
<td>36.3</td>
</tr>
<tr>
<td>0.45</td>
<td>21.4</td>
<td>39.1</td>
<td>31.2</td>
</tr>
<tr>
<td>0.50</td>
<td>32.6</td>
<td>31.1</td>
<td>21.3</td>
</tr>
<tr>
<td>0</td>
<td>0.64</td>
<td>0.64</td>
<td>1.07</td>
</tr>
</tbody>
</table>

* Not corrected for titration of phosphate (\(PO_4\)) in media.
Table XX

Yields of Acid and Mycelium Produced with Varying Amounts of Sucrose

<table>
<thead>
<tr>
<th>Gms. of Sucrose per 100 cc of media</th>
<th>cc of N/10 base per 10 cc of acid</th>
<th>Wt. of mycelium per 100 cc</th>
</tr>
</thead>
<tbody>
<tr>
<td>3rd day</td>
<td>5th day</td>
<td>9th day</td>
</tr>
<tr>
<td>1</td>
<td>1.71</td>
<td>1.7</td>
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<td>2</td>
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<td>2.2</td>
</tr>
<tr>
<td>4</td>
<td>4.5</td>
<td>2.8</td>
</tr>
<tr>
<td>5</td>
<td>4.1</td>
<td>6.7</td>
</tr>
<tr>
<td>6</td>
<td>5.6</td>
<td>11.9</td>
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<td>17.2</td>
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<td>15</td>
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<td>15.8</td>
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<tr>
<td>18</td>
<td>9.4</td>
<td>17.1</td>
</tr>
<tr>
<td>19</td>
<td>6.7</td>
<td>10.5</td>
</tr>
</tbody>
</table>

Summary of the Studies on the Effect of the Composition of the Media on the Growth of Aspergillus niger

From the preceding data one may readily see that the composition of media may be varied somewhat and still produce extremely good growth along with high yields of acid. However, the medium which would probably give the highest yields of acid would have approximately the following composition per liter: Sucrose - 100 to 125 grams, potassium
dihydrogen phosphate - 1 to 1.5 grams, ferrous sulfate - 0.95 to 0.15 gram, zinc sulfate 0.05 to 0.15 gram, magnesium sulfate - 0.7 to 1 gram, ammonium chloride - 1.75 to 3.35 grams. In order to get slightly higher yields of mycelium one can increase just a little the zinc and iron and the ammonium chloride.

It must be borne in mind too that the surface volume ratio is of much importance. Much better results were obtained using 100 cc of medium in 500 cc flasks than in using 500 cc of media in two liter Erlenmeyer flasks. In the first case, one has a total surface area of about 44.8 square cm. In the latter case, about 173 sq. cm. of surface area. If one divides each surface by the volume of the liquid under it, one has the surface volume ratio. One has then, 44.8/100 or 0.448 as the surface volume ratio for the small flask and 173/500 or 0.358 for the two liter flasks. However, 0.448 may not be the best surface volume ratio for there is not sufficient data to determine that definitely.
THE NATURE OF THE MATERIALS PRODUCED BY MOLDS

THE STIMULAT VE YOAST GROWTH

It has been previously shown that a stimulative material has been produced by each of the several molds studied. Of these molds, however, the two, *Aspergillus niger* and *Aspergillus clavatus* grew the most rapidly and produced a comparatively very high yield of the "bios". Therefore, these two molds were chosen to do further work with; namely, to attempt to study the material or materials produced by them that seem to stimulate yeast. The object being to grow these molds in sufficient quantities that both the composition of the media on which they had grown and the composition of their mycelia could be studied.

For a study of the stimulant remaining in the media or the extra-cellular stimulant it was found to be more desirable to use the glycerine medium because the sucrose remaining behind after the mold had grown made further fractionation and purification very difficult. In each instance the composition of the media was that previously indicated by the Medium J of Palmer, Nelson, and Swarbrick, or the modified media of Czapek used by Hoyer, Weisbrot-Smith, and Collins (1930) and will for simplicity be merely designated by the sucrose medium and the glycerine medium respectively.

The procedure in general was as follows: The medium was prepared as indicated, sterilized in 500 c.c. quantities
in two liter Erlenmeyer flasks and cooled and inoculated with the desired cultures and incubated at room temperature for fourteen days. The mycelium was then removed by filtration, washed with distilled water and urine, and the filtrate used for further fractionation and study. In this way a large volume of filtrate was obtained as well as a considerable quantity of mycelium.

A STUDY OF THE VOLATILITY FROM ASPERGILLUS

The Volatility of the Stimulant

One liter of the *Aspergillus clavatus* filtrate from glycerine medium was distilled until 100 c.c. of solution remained. The distillate was saved as well as the residue. Now, if the stimulant is volatile, it should be found in the distillate, if non-volatile, it should be found in the residue for it has been previously shown that the material is quite stable to heat. Therefore, the following series of media were prepared using the distillate and the residue:

1. Using nutrient salts in the proper proportions to prepare the original glycerine medium plus 30 grams of sucrose, 800 c.c. of medium were prepared using the distillate as a solvent.

2. The 100 c.c. of residue contain sufficient nutrient salts for one liter of the glycerine medium if they have not been used up by the molds. Therefore, 80 c.c. of this residue were
enriched with 50 grams of sucrose and made up to one-half liter with distilled water.

5. It might be possible that there is more than one substance responsible for the stimulation, and that part of the stimulant might be volatile and part non-volatile, so using 50 c.c. of the residue plus 27½ grams of sucrose, plus enough distillate to make 250 c.c., one has then a combination of all the possible stimulants.

6. The original hold filtrate made up to 10% concentration of sucrose. This is to check if there is actually a stimulant present.

7. The glycerine medium made up to 10% sucrose to serve as a check on yeast growth.

Summarized, the purpose of the five media are as follows:

A. Checks the stimulant in the distillate.

B. Checks the stimulant in the residue.

C. Detects whether or not the stimulant is a combination of volatile and non-volatile substances.

D. Checks if there is a stimulant.

E. Checks the yeast growth.

The above media were prepared and placed in 50 c.c. quantities in 125 c.c. Erlenmeyer flasks and sterilized with steam and incubated with Saccharomyces cerevisiae to a count of one, and incubated for 48 hours at 30 degrees and the count taken. This is the usual procedure.
The data are given in Table XVI

**Table XVI**

The Relative Stimulative Properties of the Residue and Distillate from Aspergillus glaucus on Glycerine

<table>
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<tr>
<th>Fraction Being Tested</th>
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<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
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<td>3.55</td>
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<td>6.9</td>
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<tr>
<td><strong>Adjusted to</strong></td>
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<td>6.9</td>
<td>6.9</td>
<td>6.9</td>
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<table>
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<td>99</td>
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</table>

<table>
<thead>
<tr>
<th>Average Count</th>
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<th>98</th>
<th>96</th>
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<tbody>
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<td><strong>Final pH</strong></td>
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<td>3.35</td>
<td>2.35</td>
<td>3.05</td>
<td>3.1</td>
</tr>
</tbody>
</table>

From the preceding data it is evident that the stimulant is non-volatile and is not destroyed by boiling, therefore, it should be possible to concentrate it by evaporation of its solution and thus obtain it in a form for further study and possible identification.

**Concentration of the Stimulant**

Eight liters of Aspergillus glaucus filtrate from the glycerine medium were evaporated to a small volume on a steam plate and then to almost complete dryness on a water
bath. About 30 grams of a dark somewhat grumy mess remained. This was washed thoroughly with 95% alcohol and about 55 grams of crystalline material remained behind in the alcohol. The original residue was designated as "A", the insoluble material as "B", and the alcohol soluble material as "C". A small quantity of the alcoholic solution was evaporated to dryness, the residue so obtained tested along with fractions "A" and "B" for their bios activities.

In testing the activity of the various soluble fractions the following procedure was used: The material to be tested was weighed and dissolved or suspended in water generally to 1% concentration. Then medium A, which was used as the standard medium for most of the yeast work, was prepared in such concentration that 45 c.c. of thin solution contained sufficient nutrient salts for 50 c.c. of medium B. This medium was then measured out in 45 c.c. quantities in 125 c.c. Erlenmeyer flasks and sterilized. After sterilization, the sterilized material to be tested was added in varying amounts to these flasks and the total volume made up with distilled water to 50 c.c. The media were then inoculated with yeast in the usual way. The data for the three fractions are given in Table XVII. It is clearly indicated from these data that the stimulant is concentrated in the alcohol soluble fraction. However, very likely there would be present some inorganic salts from the media as well. To remove as much of these as possible the alcoholic solution was concentrated to about 500 c.c., and an equal volume of ether added. A copious whitish precipitate formed which weighed about 6 c.c.
7 grams. The liquid was evaporated to dryness on the water bath and a residue of about 6 grams of tarry material obtained. The material precipitated by ether will be indicated by "E", while the material soluble in the other alcohol mixture will be designated by "D". The potency of these two fractions was tested as before and the data are given in Table XVII.

**Table XVII**

Fractionation of Stimulant by Means of Alcohol

<table>
<thead>
<tr>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>( \text{Check} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{mg.} \text{St.} )</td>
<td>( \text{Count} )</td>
<td>( \text{mg.} \text{St.} )</td>
<td>( \text{Count} )</td>
<td>( \text{mg.} \text{St.} )</td>
</tr>
<tr>
<td>2</td>
<td>14</td>
<td>2</td>
<td>14</td>
<td>3</td>
</tr>
<tr>
<td>10</td>
<td>26</td>
<td>10</td>
<td>17</td>
<td>10</td>
</tr>
<tr>
<td>20</td>
<td>40</td>
<td>20</td>
<td>19</td>
<td>20</td>
</tr>
<tr>
<td>40</td>
<td>92</td>
<td>40</td>
<td>22</td>
<td>40</td>
</tr>
<tr>
<td>60</td>
<td>91</td>
<td>60</td>
<td>32</td>
<td>60</td>
</tr>
<tr>
<td>80</td>
<td>100</td>
<td>80</td>
<td>42</td>
<td>80</td>
</tr>
</tbody>
</table>

A is original residue after evaporation.

B is alcohol insoluble material.

C is alcohol soluble material.

D is original media that has not supported solid growth plus 10% sucrose for check.

* \( \text{mg.} \text{St.} \) is milligrams of solid stimulant concentrate per 100 c.c. of media.*
TABLE XVIII
Further Fractionation of Alcohol Soluble Material with Ether

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Count</th>
<th>Fraction</th>
<th>Count</th>
<th>Checks</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>38</td>
<td>2</td>
<td>140</td>
<td>29</td>
</tr>
<tr>
<td>10</td>
<td>21</td>
<td>10</td>
<td>197</td>
<td>28</td>
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<tr>
<td>20</td>
<td>33</td>
<td>20</td>
<td>220</td>
<td>25</td>
</tr>
<tr>
<td>40</td>
<td>32</td>
<td>40</td>
<td>179</td>
<td>29</td>
</tr>
<tr>
<td>60</td>
<td>23</td>
<td>60</td>
<td>164</td>
<td>24</td>
</tr>
<tr>
<td>80</td>
<td>36</td>
<td>80</td>
<td>194</td>
<td>32</td>
</tr>
</tbody>
</table>

*mg. St.* is milligrams of equivalent per 100 c.c. of ether.

It has been shown by the data in Table XVIII that the stimulant is present in its highest concentration in the residue from the ether alcohol solution. Consequently, this fraction was used for further purification. A 2 gram sample of this fraction was boiled with sorbitol, then filtered and evaporated very carefully to dryness. A clear syrupy material considerably more viscous than glycerine remained. This residue weighed about 1 1/2 grams. This material retained almost the full potency of the original material as is shown by the data in Table XIX.

TABLE XIX
Effect of Sorbitol on Alcohol Ether Soluble Fraction

<table>
<thead>
<tr>
<th>Before Decoloring</th>
<th>After Decoloring</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Lg. St. *</td>
<td>Count</td>
</tr>
<tr>
<td>2</td>
<td>70</td>
</tr>
<tr>
<td>10</td>
<td>144</td>
</tr>
<tr>
<td>20</td>
<td>176</td>
</tr>
<tr>
<td>40</td>
<td>173</td>
</tr>
<tr>
<td>60</td>
<td>183</td>
</tr>
<tr>
<td>80</td>
<td>183</td>
</tr>
</tbody>
</table>
It has been contended by other workers (Wildiers, 1901) that Bosis is not soluble in strong alcohol, but is soluble in dilute alcohol. Addition of absolute alcohol had little effect on the decolorized material. Consequently, some of this material which had been purified by merits, was dissolved in just a very little water (it is very soluble) and about 300 c.c. of absolute alcohol added and shaken thoroughly. A white somewhat granular material separated which weighed about 0.7 of a gram (one gram of material was started with), and on evaporating the alcohol solution to dryness, about 0.25 gram of the whitish crystal material remained. The activity of the two fractions was tested in the usual way and it was found that the stimulant could be prepared in a considerably purer form by the use of the very strong alcoholic solutions. This is shown by the data in Table XX.

**Table XX**

<table>
<thead>
<tr>
<th>100 c.c. of media</th>
<th>A*</th>
<th>B*</th>
<th>Checks</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>132</td>
<td>38</td>
<td>26</td>
</tr>
<tr>
<td>10</td>
<td>186</td>
<td>41</td>
<td>27</td>
</tr>
<tr>
<td>20</td>
<td>195</td>
<td>50</td>
<td>21</td>
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<tr>
<td>40</td>
<td>205</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>195</td>
<td>42</td>
<td></td>
</tr>
<tr>
<td>80</td>
<td>167</td>
<td>43</td>
<td></td>
</tr>
</tbody>
</table>

* A = yeast counts for the fraction insoluble in absolute alcohol.

B = yeast counts for the fraction soluble in absolute alcohol.
Stability of the Stimulant

A one gram sample of the material soluble in the other alcohol solution was boiled with 10% KOH for 15 minutes then neutralized with HCl and diluted to 100 c.c. In a similar way a one gram sample was boiled with HCl solution made by adding 5 c.c. of concentrated HCl to 45 c.c. of water, for 15 minutes, then neutralized with KOH. The potency of these two preparations was then tested in the usual way. The results are given in table XXI.

<table>
<thead>
<tr>
<th>mg. of substance per 100 c.c.</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>19</td>
<td>31</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>34</td>
<td>55</td>
<td>48</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>42</td>
<td>65</td>
<td>65</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>49</td>
<td>85</td>
<td>99</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>61</td>
<td>104</td>
<td>152</td>
<td>17</td>
</tr>
<tr>
<td>80</td>
<td>104</td>
<td>149</td>
<td>148</td>
<td>19</td>
</tr>
<tr>
<td>100</td>
<td>17</td>
<td>19</td>
<td>16</td>
<td></td>
</tr>
</tbody>
</table>

A represents yeast counts for the material which was boiled with KOH.

B represents the yeast counts for the material which was boiled with HCl when used in the various indicated concentrations.

C represents the yeast counts in medium C under the same conditions, which serves as a check.

From the data in the preceding table it is evident that the material which is active in the stimulant is quite stable to acid and to alkali. This does not seem to be
entirely in accord with Wildiers who described (1901) his as being stable to dilute acid but changed by boiling in one percent NaOH. Therefore, it might be reasonable to assume that the material with which we are working is not the same as that with which Wildiers worked.

The Optimum Concentration of the Stimulant

In an effort to determine the optimum concentration of the material, a quantity of the material soluble in the ether alcohol mixture and decolorized by norite was added to medium C in varying concentrations to form a series of flasks. These were inoculated in the usual way and the counts taken in 48 hours. The data are indicated in table XXII.

**TABLE XXII**

Optimum Concentration of Stimulant Present

In Alcohol-Ether Fraction Decolorized by Norite

<table>
<thead>
<tr>
<th>Mr. of Material</th>
<th>Count per Ag.</th>
<th>Count of Stimulant</th>
<th>Checks</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>77</td>
<td>38.5</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>107</td>
<td>5.35</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>124</td>
<td>4.6</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>216</td>
<td>2.85</td>
<td></td>
</tr>
<tr>
<td>80</td>
<td>312</td>
<td>2.85</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>330</td>
<td>2.20</td>
<td></td>
</tr>
<tr>
<td>200</td>
<td>336</td>
<td>1.19</td>
<td></td>
</tr>
<tr>
<td>400</td>
<td>328</td>
<td>0.87</td>
<td></td>
</tr>
<tr>
<td>600</td>
<td>337</td>
<td>0.328</td>
<td></td>
</tr>
<tr>
<td>800</td>
<td>331</td>
<td>0.876</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>20</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>23</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>22</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>25</td>
</tr>
</tbody>
</table>
From the preceding table it may be seen that the optimum concentration of the stimulant in this degree of purification is about 30 milligrams per 100 c.c.

**Study of the Filtre from Aspergillus niger Grown on Glycerine Media**

Twenty liters of filtrate of *Aspergillus niger* grown on glycerine media were evaporated to dryness on a steam bath. About 180 grams of a somewhat dirty brown mass were obtained. This was fractionated in the same way that the material from the *Aspergillus clavatus* filtrate had been fractionated. The various fractions were designated as follows:

- **Fraction A.** The original residue. Yield 180 grams.
- **Fraction B.** Material insoluble in alcohol. Yield 30 grams.
- **Fraction C.** Alcohol soluble material. Yield 70 grams.
- **Fraction D.** Thrown out of the alcohol solution by ether. Yield 25 grams.
- **Fraction E.** Alcohol ether soluble material. Yield 45 grams.
- **Fraction F.** Fraction E decolorized with acetic acid.
- **Fraction G.** Fraction of F, insoluble in absolute alcohol.
- **Fraction H.** Fraction of F, soluble in absolute alcohol.

The stimulative properties of these various fractions were tested the usual way and the data are given in Table XXIII.
TABLE XIII
Yeast Counts for the Various Fractions Obtained
from Aspergillus niger Filtrate Grown on Glycerine

<table>
<thead>
<tr>
<th>mg. of Stimulant</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
<th>H</th>
<th>Checks</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>16</td>
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<td>-</td>
<td></td>
</tr>
<tr>
<td>1.0</td>
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<td>-</td>
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<td>-</td>
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<td></td>
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<tr>
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<td>67</td>
<td>37</td>
<td>88</td>
<td>21</td>
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<tr>
<td>10.0</td>
<td>18</td>
<td>14</td>
<td>185</td>
<td>13</td>
<td>157</td>
<td>103</td>
<td>59</td>
<td>103</td>
<td>55</td>
</tr>
<tr>
<td>20.0</td>
<td>21</td>
<td>21</td>
<td>185</td>
<td>14</td>
<td>155</td>
<td>133</td>
<td>115</td>
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<td>53</td>
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<tr>
<td>25.0</td>
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<td>-</td>
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</tr>
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<td>165</td>
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</tr>
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<td>17</td>
<td>178</td>
<td>167</td>
<td>195</td>
<td>195</td>
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</tr>
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<td>197</td>
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<td>-</td>
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<td>-</td>
<td>-</td>
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<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>-</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

The data in the preceding table are quite similar to the data obtained with Aspergillus clavatus and it is possible that the material or materials producing stimulation are the same in both cases.

Further Fractionation of the Stimulant

The filtrate from two 500 c.c. quantities of glycerine media that had supported the growth of Aspergillus niger for two weeks was made just alkaline with ammonium hydroxide, then
lead acetate added as long as a precipitate formed. The opaque yellowish precipitate thus obtained was removed and suspended in water, acidified with HCl and the lead removed with hydrogen sulfide. The lead was removed from the filtrate in the same way. The two fractions thus obtained were saved for further work.

Another one liter portion of the same filtrate was treated with barium hydroxide as long as a precipitate could form, the material was filtered and the barium removed from both the filtrate and precipitate with sulfuric acid.

It was assumed that sufficient nutrient salts were still present in the solutions of the filtrate to permit the growth of yeast. But in order to get growth in solution of the precipitate it was deemed necessary to enrich these solutions with the proper concentrations of salts to make these media the same as the original medium. These four fractions were then inoculated, after sterilization, with yeast in the usual manner. It was noticed that apparently the stimulant has been completely destroyed by the lead precipitation. The material, however, was not precipitated or destroyed by barium hydroxide. The data are indicated in Table XXIV.
TABLE LXIV
Yeast Counts for the Various Fractions Obtained
by Sodium Hydroxide and Lead Acetate

<table>
<thead>
<tr>
<th>4</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
<th>H</th>
</tr>
</thead>
<tbody>
<tr>
<td>96</td>
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<td>12</td>
<td>15</td>
<td>31</td>
<td>14</td>
<td>82</td>
<td>21</td>
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<tr>
<td>108</td>
<td>14</td>
<td>15</td>
<td>14</td>
<td>75</td>
<td>19</td>
<td>83</td>
<td>19</td>
</tr>
<tr>
<td>100</td>
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<td>70</td>
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<tr>
<td>86</td>
<td>12</td>
<td>13</td>
<td>10</td>
<td>32</td>
<td>15</td>
<td>88</td>
<td></td>
</tr>
</tbody>
</table>

Yeast Counts represent quadruplicate runs on each substance tested.

Fraction A is original filtrate plus sucrose.

" B is the lead acetate precipitate.

" C is the lead acetate filtrate.

" D is barium hydroxide precipitate.

" E is barium hydroxide filtrate.

" F is mixture of filtrate and precipitate from lead acetate.

" G is a mixture of filtrate and precipitate from barium hydroxide.

" H is check.

The Production of Stimulants by Aspergillus niger as a Function of the Glycerol Concentration

In order to determine the optimum conditions for the mold growth and also to determine the optimum conditions for production of growth stimulants the following basal medium was used. AgNO₃ -- 0.4 grams, FeSO₄ -- 3.1 grams.
ZnSO₄ — 0.1 gram, KOH — 1.8 grams, NH₄Cl — 2 grams.

To this medium, glycerine was added to the various flasks in varying amounts. It was then sterilized and inoculated with *Aspergillus niger* and allowed to grow for fourteen days. The mold was removed and the filtrate enriched by adding 100 grams of sucrose per liter of solution and the pH adjusted to 6.9. It was then added to the original basic media, enriched by adding 100 grams sucrose per liter, in varying amounts. This basal or control medium was the same as the original plus 100 grams of sugar per liter.

Here lies the following variable factors:

1. The varying concentration of glycerine from which one hopes to be able to establish an optimum for the production of stimulant relative to the concentration of glycerine.

2. The varying concentration of filtrate in the media should enable one to determine an optimum concentration of filtrate. By plotting the concentration of glycerine against average count of yeast per c.c. of filtrate used, one gets the optimum concentration of glycerine for filtrate produced. (Graph No. I.) By plotting the concentration of glycerine against weight of ascospore, one gets the optimum for the production of ascospores from glycerine. (Graph No. II.) By dividing the values obtained from the average count per c.c. of glycerine filtrate by the grams of glycerine per 1000 c.c., one gets the average count per gram of glycerine used.
### Table IX

The Effect of Varying the Concentrations of Nutrients

In the Production of Stolon

<table>
<thead>
<tr>
<th>Media Number</th>
<th>Media I</th>
<th>Media II</th>
<th>Media III</th>
<th>Media IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>C.C. of control</td>
<td>C.C. of media</td>
<td>Filtrate</td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>45</td>
<td>5</td>
<td>10</td>
<td>2</td>
<td>15</td>
</tr>
<tr>
<td>40</td>
<td>10</td>
<td>15</td>
<td>1.5</td>
<td>24</td>
</tr>
<tr>
<td>25</td>
<td>20</td>
<td>24</td>
<td>0.35</td>
<td>28</td>
</tr>
<tr>
<td>20</td>
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<td>1.12</td>
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</tr>
<tr>
<td>20</td>
<td>30</td>
<td>30</td>
<td>1.0</td>
<td>62</td>
</tr>
<tr>
<td>10</td>
<td>40</td>
<td>42</td>
<td>1.05</td>
<td>61</td>
</tr>
<tr>
<td>0</td>
<td>50</td>
<td>39</td>
<td>0.78</td>
<td>101</td>
</tr>
</tbody>
</table>

**Average count per c.c. of filtrate:**

<table>
<thead>
<tr>
<th>Media Number</th>
<th>Media V</th>
<th>Media VI</th>
<th>Media VII</th>
</tr>
</thead>
<tbody>
<tr>
<td>C.C. of control</td>
<td>C.C. of media</td>
<td>Filtrate</td>
<td>A</td>
</tr>
<tr>
<td>45</td>
<td>5</td>
<td>57</td>
<td>19.4</td>
</tr>
<tr>
<td>40</td>
<td>10</td>
<td>103</td>
<td>10.5</td>
</tr>
<tr>
<td>25</td>
<td>20</td>
<td>110</td>
<td>3.05</td>
</tr>
<tr>
<td>20</td>
<td>25</td>
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<td>4.10</td>
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<tr>
<td>20</td>
<td>30</td>
<td>99</td>
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<td>10</td>
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</tr>
<tr>
<td>0</td>
<td>50</td>
<td>51</td>
<td>1.02</td>
</tr>
</tbody>
</table>

**Average count per c.c. of filtrate:**

<table>
<thead>
<tr>
<th>Media Number</th>
<th>Media V</th>
<th>Media VI</th>
<th>Media VII</th>
</tr>
</thead>
<tbody>
<tr>
<td>Filtrate</td>
<td>A</td>
<td>B</td>
<td>A</td>
</tr>
</tbody>
</table>

*$a$ = average count.

$**b**$ = count per c.c. of filtrate.
Graph I
From data pages 31 and 32

Concentration of glycerol per L

Graph II
From data page 51

Concentration of glycerol per L

All of my data peratures of 0.0
The data are given in Tables XXV and XXVI

**Table XXVI**

Table Showing Yields of Acid and Mycelium by
Aspergillus niger on Varying Amounts of Glycerine

<table>
<thead>
<tr>
<th>Series</th>
<th>Gas. of Glycerine a.c. of H/10</th>
<th>% of Gas. of Myc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>per 1000 c.c.</td>
<td>% e. g. per e. of Glycerin</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>2.5</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>3.0</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>3.2</td>
</tr>
<tr>
<td>4</td>
<td>20</td>
<td>3.9</td>
</tr>
<tr>
<td>5</td>
<td>35</td>
<td>3.8</td>
</tr>
<tr>
<td>6</td>
<td>50</td>
<td>2.5</td>
</tr>
<tr>
<td>7</td>
<td>60</td>
<td>4.0</td>
</tr>
</tbody>
</table>

* a.c. of H/10 base per 10 c.c. of acid at the end of a fermentation.
** Weight of mycelium dried from 500 c.c. of medium.

The Production of Stimulants by Aspergillus niger on Glycerine as a Function of Time.

In order to more definitely follow the production of stimulants by Aspergillus niger, it was deemed necessary to study the production of stimulants as a function of time. It had been previously shown that the following medium, magnesium sulfate - 0.4 gram, ferrrous sulfate - 0.1 gram, zinc sulfate - 0.1 gram, potassium dihydrogen phosphate - 1.55 grams, ammonium carbonate - 2.00 grams, glycerine - 40 grams, produced the maximum yield of stimulant per gram of glycerine used. This medium was sterilized in 500 c.c. quantities in two-liter Erlenmeyer flasks and inoculated with Aspergillus niger. At varying intervals, the stimulative properties of the filtrate were determined by using a flask of the medium.
# Table XVII

Production of Yeast by Aspergillus niger as a Function of Time

<table>
<thead>
<tr>
<th>Length of Hold (days)</th>
<th>5</th>
<th>7</th>
<th>9</th>
<th>11</th>
<th>14</th>
<th>18</th>
<th>24</th>
<th>50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wt. of mycelium (g)</td>
<td>0.69</td>
<td>1.71</td>
<td>3.69</td>
<td>5.03</td>
<td>5.14</td>
<td>4.34</td>
<td>3.34</td>
<td>2.84</td>
</tr>
<tr>
<td>pH</td>
<td>2.4</td>
<td>2.8</td>
<td>3.4</td>
<td>4.7</td>
<td>3.3</td>
<td>3.3</td>
<td>3.3</td>
<td>3.2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>C.C. of control</th>
<th>C.C. of mold</th>
<th>Yeast Count</th>
</tr>
</thead>
<tbody>
<tr>
<td>media</td>
<td>filtrate</td>
<td></td>
</tr>
<tr>
<td>45</td>
<td>5</td>
<td>13</td>
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<td>15</td>
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<td>41</td>
</tr>
<tr>
<td>10</td>
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<td>6</td>
<td>7</td>
</tr>
<tr>
<td>50</td>
<td>8</td>
<td>9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Length of Hold (days)</th>
<th>30</th>
<th>48</th>
<th>50</th>
<th>55</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wt. of mycelium (g)</td>
<td>2.22</td>
<td>2.22</td>
<td>2.20</td>
<td>2.20</td>
</tr>
<tr>
<td>pH</td>
<td>3.4</td>
<td>3.2</td>
<td>3.2</td>
<td>3.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>C.C. of control</th>
<th>C.C. of mold</th>
<th>Yeast Count</th>
</tr>
</thead>
<tbody>
<tr>
<td>media</td>
<td>filtrate</td>
<td></td>
</tr>
<tr>
<td>45</td>
<td>5</td>
<td>34</td>
</tr>
<tr>
<td>40</td>
<td>10</td>
<td>35</td>
</tr>
<tr>
<td>30</td>
<td>20</td>
<td>72</td>
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<td>25</td>
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<td>79</td>
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<tr>
<td>20</td>
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<tr>
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<td>40</td>
<td>90</td>
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<tr>
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<td>0</td>
<td>11</td>
</tr>
<tr>
<td>50</td>
<td>0</td>
<td>10</td>
</tr>
</tbody>
</table>
enriched with sucrose to a concentration of 100 grams per
liter. The pH was also adjusted to 6.9 with sodium
hydroxide, then this medium was added in varying amounts to
the original medium which also contained 100 grams of
sucrose per liter. This enabled one to have a medium of
somewhat definite composition and yet to vary the con-
centration of the stimulant, at the same time, a 10 c.c.
sample was titrated with sodium hydroxide, and the mold
mycelium dried and weighed. It was noticed that the
concentration of stimulant appeared to be at a maximum
in about 14 to 18 days, and after that time it seemed to
lose some of its potency. The data are given in Table XXVII.

The Production of Stimulants by *Aspergillus Niger* on
Sucrose as Correlated with the Production of Acid.

Previous studies have shown the following medium to
be optimal in the production of acid by *Aspergillus Niger*
on sucrose: *Sucrose* - 100 grams, magnesium sulfate - 0.4 gram,
terrous sulfate and zinc sulfate each - 0.1 gram, ammonium
chloride - 2.00 grams, and water to one liter, pH - 6.9.
This medium was prepared and placed in 100 c.c. quantities
in 250 c.c. flasks, sterilized, and inoculated with *Aspergillus
Niger*. It has been previously shown that using a larger
volume surface ratio than the one obtained in this instance
gives poorer yields of acid. At varying intervals of
time throughout the 14 day growing period of the mold,
flasks of the material were used to study their acid content.
### TABLE XXVIII

Production of Stimulants by Aspergillus niger on Sucrose as a Function of Time and Correlated with Acid Production

<table>
<thead>
<tr>
<th>Days of Hold Fermentation</th>
<th>3</th>
<th>5</th>
<th>7</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ft. of Yeast</td>
<td>0.88</td>
<td>1.61</td>
<td>1.85</td>
<td>1.74</td>
</tr>
<tr>
<td>C.C. H/10 Base per 10 C.C.</td>
<td>4.1</td>
<td>24.9</td>
<td>58.0</td>
<td>47.5</td>
</tr>
</tbody>
</table>

<table>
<thead>
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<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
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<td>44</td>
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<td>53</td>
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<td>94</td>
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<td>10</td>
<td>49</td>
<td>53</td>
<td>63</td>
<td>52</td>
<td>55</td>
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<td>159</td>
<td>139</td>
<td>107</td>
<td>121</td>
<td>114</td>
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<td>30</td>
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<td>124</td>
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<td>82</td>
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<td>9</td>
<td>10</td>
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<table>
<thead>
<tr>
<th>Days of Hold Fermentation</th>
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<th>14</th>
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</thead>
<tbody>
<tr>
<td>Ft. of Yeast</td>
<td>2.53</td>
<td>2.65</td>
<td>2.74</td>
</tr>
<tr>
<td>C.C. H/10 Base per 10 C.C.</td>
<td>55.5</td>
<td>59.5</td>
<td>63.0</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>C.C. of Control</th>
<th>C.C. of Hold</th>
<th>Yeast Count</th>
<th>Media</th>
<th>A*</th>
<th>A*</th>
<th>A*</th>
<th>B*</th>
<th>A</th>
<th>B</th>
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<tbody>
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<td>32</td>
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<td>88</td>
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<tr>
<td>40</td>
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<td>11</td>
<td>14</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

A* Media sterilized by steam.
B* Media sterilized by filtration.
To do this the filtrate from the mold was obtained in the usual way and enriched with 5 grams of sucrose per 100 c.c. to compensate for that used up by the mold. This new filtrate was then added in varying amounts to the original medium in which no mold had grown. These flasks of media were then sterilized by steam at first. However, this was not so successful because the invert sugar from the mold filtrate charred badly. Consequently, the material was sterilized through a Buchner filter before mixing. The data indicate that the concentration of stimulants reaches a maximum within 3 to 5 days after inoculation with the mold. After that time there appears to be a gradual decrease in the concentration of the stimulants. Also, there is some evidence that the filtrate becomes toxic or at any rate much less stimulative in higher concentrations in the older flasks of medium. The data are given in Table LXXI.

The Production of a Stimulant of an Intracellular Nature

**by Aspergillus niger.**

The previous data indicate and describe an extra-cellular product. It seemed of interest to test the mold itself. A 200 gram sample of dried Aspergillus niger mycelium that had been grown on sucrose medium, was extracted for 20 hours with 2 liters of water at 20 to 60 degrees. The suspension was filtered and evaporated to a thick syrupy mass in vacuo at 30 degrees and dried in a desiccator over calcium chloride. The resulting material was quite black.
and dough-like in consistency and amounted to about 30 grams.

This was designated as Fraction I. A 25 gram sample of this fraction was continuously extracted for four days with 95% alcohol. The extract in contact with the mold was greenish in color while that in the reservoir was reddish brown. On concentrating the alcoholic extract and cooling it to minus 10 degrees a considerable quantity of needle-like crystals separated, together with some tarry material. The alcohol insoluble material was designated as Fraction II. The alcohol soluble material not removed by cooling was Fraction III. The material separated by cooling is Fraction IV. The effects of these four fractions on the growth of yeast at 30 degrees in Medium C are shown in Table XXX.

It will be noticed from the data in Table XXX that Fractions I, II, III, and IV are very rich in the stimulant.

Table XXX


<table>
<thead>
<tr>
<th>Concentration</th>
<th>Count</th>
<th>Concentration</th>
<th>Count</th>
<th>Concentration</th>
<th>Count</th>
<th>Concentration</th>
<th>Count</th>
<th>Concentration</th>
<th>Count</th>
</tr>
</thead>
<tbody>
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<td>0</td>
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<td>0</td>
<td>60</td>
<td>0</td>
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<td>0</td>
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<td>58</td>
<td>0</td>
<td>57</td>
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<td>67</td>
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</tr>
<tr>
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<td>55</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
cells per cubic centimeter

\[ \text{Count} = \frac{C}{250,000} \]

I. Water-soluble fraction, pH of 1% solution = 4.4

II. Alcohol-insoluble fraction, pH of 1% solution = 3.7

III. Alcohol-soluble fraction, pH of 1% solution = 3.3

IV. Material removed from alcohol-soluble fraction by cooling to minus 10 degrees C, pH of 1% solution = 3.7

Relationship between Sios I and Sios II of Miller and the material of aios activity described by Taha.

According to Dr. Miller and co-workers (J. Am. C. H. S., 1937) the water soluble extract of malt sprouts can be divided into two separate constituents by a series of hydroxides.

The one, Sios I, is precipitated by a series of hydroxides and is non-stimulative, and the other, Sios II, which is not precipitated by a series of hydroxides, is only slightly stimulative alone, but combinations of I and II are highly stimulative.

Sios I has been identified as inositol by Lister (1938).

Sios I and Sios II were prepared according to Miller's directions and made up in concentration five times that assayed by him. In so doing, it was possible to use 1 cc. in 60 cc. of media and have the same concentration as Miller did with 1 cc. in 10 cc. in his paper. The first several attempts to prepare Sios I and II did not yield satisfactory results. Generally both the separate fractions, Sios I and II, were quite highly stimulative even though Miller's directions were followed with great pains. The most satisfactory preparation according to results obtained gave the following counts. The stimulative properties of inositol were tested along with Sios I and Sios II. See Table XXX.

* The malt sprouts were kindly furnished through Mr. C. S. Hiner, of the Minor laboratories.
### Table XXX

The Relationship between Bios I and II of Miller and the Stimulant Obtained from aspergillus clavatus filtrate

<table>
<thead>
<tr>
<th>Acid (pH)</th>
<th>Bios I</th>
<th>Bios II</th>
<th>Inositol**</th>
<th>Count</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>1 c.c.</td>
<td>1 c.c.</td>
<td>114</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>1 c.c.</td>
<td>1 c.c.</td>
<td>151</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>1 c.c.</td>
<td>1 c.c.</td>
<td>151</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>1 c.c.</td>
<td>1 c.c.</td>
<td>147</td>
<td></td>
</tr>
<tr>
<td>80</td>
<td>1 c.c.</td>
<td>1 c.c.</td>
<td>148</td>
<td></td>
</tr>
</tbody>
</table>

Check Medium 2

* Stimulant from aspergillus clavatus on glycerine after decolorizing with nitrite. Concentration in milligrams per 100 c.c.

** Inositol from Eastman Kodak Company. Concentration mg. per 100 c.c.
It was considered of interest to see whether or not the B1s obtained from A. alcalia clading which was soluble in alcohol-water mixture bore any relationship to the B1s I and II of Miller. Consequently, a series of bleks was prepared using Medium J. To these bleks a solution of the solid B1isms was added along with B1s I or B1s II in varying concentrations. The relationship to inositol was tested also. The solid fraction itself is quite potent and its stimulative qualities are increased by addition of B1s II. Inositol and B1s I have no appreciable effect. The data are indicated in Table XXX.

The A. alcalia extracts of Palmer, Holm, and Scheeler, and their Relationship to B1s I and B1s II of Miller.

Palmer, Holm, and Scheeler (1932) separated the water soluble extract of A. alcalia into four fractions by fractional precipitation with alcohol, and with these four fractions proved the multiple nature of B1s.

Williams (1921) gives Knepper credit for the original discovery of B1s, but goes further and gives Nash Miller credit for having discovered an entirely new system of B1isms from malt sprouts, namely, B1s I and B1s II. It seems absurd that each yeast or each variety of yeast should have a specific B1s or B1isms. It would seem much more logical to believe that there are many things that might function as a B1s. Perhaps too, B1s might really be a "set of conditions". In other words, then one produces optimum conditions with respect to maximal B1s, pH,
oxidation-reduction potential, one then has optimum conditions and, consequently, maximal growth, and that such a thing as bios or elixir of life might be arrived in the same category as Philosopher's Stone as a thing beautiful to think about but absurd to practical minds.

If the four fractions from alfalfa as obtained by Palmer and co-workers cannot be separated into bios I and II by Miller's method, and if addition of bios I and II separately to each of these four fractions does not produce enhanced growth, it would be logical to conclude that the stimulative material was something other than bios I and bios II of Miller, and that four new substances had been added to the already large number of materials with bios properties.

Starting with 1600 grams of alfalfa and following carefully the method of Palmer, Nelson, and Ercolani the four fractions were obtained as designated by them. The yields were as follows:

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraction I</td>
<td>47.9 grams</td>
</tr>
<tr>
<td>Fraction II</td>
<td>64 grams</td>
</tr>
<tr>
<td>Fraction III</td>
<td>210 grams</td>
</tr>
<tr>
<td>Fraction IV</td>
<td>132 grams</td>
</tr>
</tbody>
</table>

These were used in yeast nutrition studies in the usual manner. The optimum concentration of each was found. Bios I and bios II are insoluble added to each at the optimum concentration, and Fractions III and IV subjected to Miller's method of fractionation for bios I and II.
The Alfalfa Fractions of Pulver, Nelson, and Blacker

T able XXXI

<table>
<thead>
<tr>
<th>Yeast Counts</th>
<th>Fraction I</th>
<th>Fraction II</th>
<th>Fraction III</th>
<th>Fraction IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>12</td>
<td>12</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td>0.5</td>
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<td>18</td>
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<tr>
<td>2.5</td>
<td>31</td>
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<td>31</td>
<td>31</td>
</tr>
<tr>
<td>5.0</td>
<td>55</td>
<td>55</td>
<td>55</td>
<td>55</td>
</tr>
<tr>
<td>10.0</td>
<td>67</td>
<td>67</td>
<td>67</td>
<td>67</td>
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<tr>
<td>15.0</td>
<td>88</td>
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<td>88</td>
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<tr>
<td>20.0</td>
<td>105</td>
<td>105</td>
<td>105</td>
<td>105</td>
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<tr>
<td>25.0</td>
<td>123</td>
<td>123</td>
<td>123</td>
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<tr>
<td>30.0</td>
<td>153</td>
<td>153</td>
<td>153</td>
<td>153</td>
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<tr>
<td>35.0</td>
<td>183</td>
<td>183</td>
<td>183</td>
<td>183</td>
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<tr>
<td>40.0</td>
<td>214</td>
<td>214</td>
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<tr>
<td>45.0</td>
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<td>243</td>
<td>243</td>
<td>243</td>
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<tr>
<td>50.0</td>
<td>274</td>
<td>274</td>
<td>274</td>
<td>274</td>
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<tr>
<td>55.0</td>
<td>304</td>
<td>304</td>
<td>304</td>
<td>304</td>
</tr>
<tr>
<td>60.0</td>
<td>333</td>
<td>333</td>
<td>333</td>
<td>333</td>
</tr>
</tbody>
</table>

Taking the optimum concentration of these four fractions
(Table XXXI and XXXII) and combining the various fractions using
the optimum conditions, one gets increased stimulation over
the original optimum for the separate fractions. This gives
one evidence again of the multiple nature of the stimulant
present.

The data obtained by adding Miller's Bios I and II to
the four alfalfa fractions (Table XXXII) are somewhat confusing.
It will be noticed that there is increased stimulation when
Bios I is added to fractions I, III, and IV of the alfalfa
extract while there is not so much stimulation noticed with
Bios I and Fraction II. It would be logical to assume that
Fractions I, III, and IV were deficient in Bios I. However,
a considerable stimulation is also obtained with fractions
of fractions III and IV and vice II which would indicate that
these two fractions were deficient in fraction II which is unlikely.
It seems more logical to believe that the material or
mATERIALS present in fraction I and IV and the four smaller
fractions may be of an entirely different nature, and the in-
creased stimulation is merely due to the addition of larger
quantities of a variety of stimulants in the media. It is fur-
ther noticed that the attempted fractionation of mixture fractions
III and IV into I and II, according to Miller's directions,
was unsuccessful. In this fractionation no precipitate was
obtained in both cases with barium hydroxide, but successively
it was some other substance than Ios I. (Table XIV).
An attempted fractionation of the media of amycillinae mix
Into Mix. I and II.

One hundred grams of thoroughly ground and dried spores
from amycillinae mix grown on sucrose were extracted with
warm water and fractionated by means of barium hydroxide
according to Miller's directions. (Jnac., v. 9. (1924)).
Only a slight precipitate formed with the mixture fraction.
However, the two fractions so obtained were carried through
with the usual procedure and made up in concentration.
five times the concentration specified by Imac. (29.4).
This enabled one to add 1 c.c. of this material to 10 c.c.
of water and have the same concentration as Imac had with
his I c.c. in 10 c.c. of his dyes preparations from salt mixture.
It will be seen that this attempted fractionation was
unsuccessful. Fraction I proves to be definitely toxic in higher concentrations. Fraction II is somewhat stimulative but the combination of I and II does not produce great stimulation. Further, the addition of Miller's fractions from malt sprouts to these fractions from the solids does not give greatly enhanced growth showing that apparently they are not rich in the respective bases initiated. See Table XXXV.

**Table XXXV**

**Yeast Counts for the Optimum Concentrations of the Four Miller Fractions and for the Various Combinations of Them.**

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Yeast Count***</th>
<th>Ash. Minerals</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>II</td>
<td>III</td>
</tr>
<tr>
<td>30%</td>
<td>72</td>
<td>77</td>
</tr>
<tr>
<td>30</td>
<td>117</td>
<td>143</td>
</tr>
<tr>
<td>40</td>
<td>124</td>
<td>117</td>
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<tr>
<td>50</td>
<td>32</td>
<td>106</td>
</tr>
<tr>
<td>20</td>
<td>33</td>
<td>93</td>
</tr>
<tr>
<td>30</td>
<td>113</td>
<td>104</td>
</tr>
<tr>
<td>40</td>
<td>173</td>
<td>163</td>
</tr>
<tr>
<td>50</td>
<td>167</td>
<td>164</td>
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<td>30</td>
<td>150</td>
<td>134</td>
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<td>40</td>
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<td>60</td>
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<tr>
<td>30</td>
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<tr>
<td>40</td>
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<td>115</td>
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<tr>
<td>50</td>
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<td>30</td>
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<td>134</td>
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<td>40</td>
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<td>40</td>
<td>155</td>
<td>115</td>
</tr>
<tr>
<td>50</td>
<td>158</td>
<td>158</td>
</tr>
</tbody>
</table>
**TABLE XXXII (Continued)**

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Yeast Count**</th>
<th>Ave. Check***</th>
</tr>
</thead>
<tbody>
<tr>
<td>I II III IV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 20</td>
<td>202 198 193 203 23</td>
<td></td>
</tr>
<tr>
<td>30 30</td>
<td>316 288 258 308 21</td>
<td></td>
</tr>
<tr>
<td>40 40</td>
<td>256 189 191 138 22</td>
<td></td>
</tr>
<tr>
<td>50 50</td>
<td>386 280 266 240 24</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Yeast Count**</th>
<th>Ave. Check***</th>
</tr>
</thead>
<tbody>
<tr>
<td>I II III IV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30 30</td>
<td>215 348 245 184 33</td>
<td></td>
</tr>
<tr>
<td>30 30</td>
<td>196 233 223 251 21</td>
<td></td>
</tr>
<tr>
<td>40 40</td>
<td>260 255 245 250 23</td>
<td></td>
</tr>
<tr>
<td>50 50</td>
<td>183 192 160 170 34</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Yeast Count**</th>
<th>Ave. Check***</th>
</tr>
</thead>
<tbody>
<tr>
<td>I II III IV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 20</td>
<td>272 325 360 263 22</td>
<td></td>
</tr>
<tr>
<td>30 30</td>
<td>328 288 248 246 25</td>
<td></td>
</tr>
<tr>
<td>40 40</td>
<td>270 206 240 289 25</td>
<td></td>
</tr>
<tr>
<td>50 50</td>
<td>222 190 190 302 24</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Yeast Count**</th>
<th>Ave. Check***</th>
</tr>
</thead>
<tbody>
<tr>
<td>I II III IV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 20</td>
<td>277 277 268 275 26</td>
<td></td>
</tr>
<tr>
<td>30 30</td>
<td>245 250 258 250 21</td>
<td></td>
</tr>
<tr>
<td>40 40</td>
<td>270 333 369 237 23</td>
<td></td>
</tr>
<tr>
<td>50 50</td>
<td>225 280 260 246 24</td>
<td></td>
</tr>
</tbody>
</table>

* Yeast count in basal medium 2 for check
** Milligrams of dried alfalfa fraction per 100 c.c. of seed.
*** Yeast counts in flasks containing stimulant, run in triplicate.

**TABLE XXXII**

Yeast Counts for Optimal Concentrations of the Four Alfalfa Fractions plus Bic. I and II of Miller.

<table>
<thead>
<tr>
<th>Fractions from Alfalfa</th>
<th>Yeast Counts**</th>
<th>Ave. Check***</th>
</tr>
</thead>
<tbody>
<tr>
<td>I II III IV Bic I Bic II</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Yeast Count**</th>
<th>Ave. Check***</th>
</tr>
</thead>
<tbody>
<tr>
<td>I II III IV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20***</td>
<td>1 c.c.</td>
<td>190 176 170 172 34</td>
</tr>
<tr>
<td>20</td>
<td>2 c.c.</td>
<td>224 218 205 213 22</td>
</tr>
<tr>
<td>40</td>
<td>1 c.c.</td>
<td>280 250 213 232 25</td>
</tr>
<tr>
<td>40</td>
<td>2 c.c.</td>
<td>216 289 220 225 24</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Yeast Count**</th>
<th>Ave. Check***</th>
</tr>
</thead>
<tbody>
<tr>
<td>I II III IV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>1 c.c.</td>
<td>53 61 52 58 17</td>
</tr>
<tr>
<td>20</td>
<td>2 c.c.</td>
<td>70 74 60 70 19</td>
</tr>
<tr>
<td>40</td>
<td>1 c.c.</td>
<td>77 83 91 89 22</td>
</tr>
<tr>
<td>40</td>
<td>2 c.c.</td>
<td>76 82 70 72 -</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Yeast Count**</th>
<th>Ave. Check***</th>
</tr>
</thead>
<tbody>
<tr>
<td>I II III IV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>1 c.c.</td>
<td>176 190 184 176 17</td>
</tr>
<tr>
<td>40</td>
<td>2 c.c.</td>
<td>179 187 185 177 19</td>
</tr>
<tr>
<td>50</td>
<td>2 c.c.</td>
<td>175 180 191 182 22</td>
</tr>
<tr>
<td>60</td>
<td>1 c.c.</td>
<td>187 193 211 197 -</td>
</tr>
<tr>
<td>Fractions from Alfalfa</td>
<td>Yeast Counts</td>
<td>Ave. Check</td>
</tr>
<tr>
<td>------------------------</td>
<td>-------------</td>
<td>------------</td>
</tr>
<tr>
<td>I (20 c.c.)</td>
<td>141 150 162</td>
<td>152 22</td>
</tr>
<tr>
<td>II (30 c.c.)</td>
<td>176 182 186</td>
<td>182 22</td>
</tr>
<tr>
<td>III (40 c.c.)</td>
<td>208 207 217</td>
<td>215 28</td>
</tr>
<tr>
<td>IV (50 c.c.)</td>
<td>255 254 257</td>
<td>257 -</td>
</tr>
<tr>
<td>I (60 c.c.)</td>
<td>1 c.c.</td>
<td>120 91 100</td>
</tr>
<tr>
<td>II (80 c.c.)</td>
<td>103 102 129</td>
<td>113 21</td>
</tr>
<tr>
<td>III (40 c.c.)</td>
<td>131 143 136</td>
<td>143 24</td>
</tr>
<tr>
<td>IV (50 c.c.)</td>
<td>155 160 155</td>
<td>157 35</td>
</tr>
<tr>
<td>I (20 c.c.)</td>
<td>1 c.c.</td>
<td>116 97 88</td>
</tr>
<tr>
<td>II (30 c.c.)</td>
<td>107 123 128</td>
<td>117 21</td>
</tr>
<tr>
<td>III (40 c.c.)</td>
<td>161 157 173</td>
<td>168 24</td>
</tr>
<tr>
<td>IV (50 c.c.)</td>
<td>150 169 176</td>
<td>175 35</td>
</tr>
<tr>
<td>I (20 c.c.)</td>
<td>1 c.c.</td>
<td>192 223 195</td>
</tr>
<tr>
<td>II (30 c.c.)</td>
<td>193 214 187</td>
<td>190 21</td>
</tr>
<tr>
<td>III (40 c.c.)</td>
<td>184 182 173</td>
<td>188 24</td>
</tr>
<tr>
<td>IV (50 c.c.)</td>
<td>183 238 215</td>
<td>219 35</td>
</tr>
<tr>
<td>I (20 c.c.)</td>
<td>1 c.c.</td>
<td>152 122 137</td>
</tr>
<tr>
<td>II (30 c.c.)</td>
<td>306 307 227</td>
<td>215 21</td>
</tr>
<tr>
<td>III (40 c.c.)</td>
<td>310 260 137</td>
<td>209 24</td>
</tr>
<tr>
<td>IV (50 c.c.)</td>
<td>211 226 206</td>
<td>215 25</td>
</tr>
</tbody>
</table>

* Rice I of Miller. Concentration five times that used by him. This gives the same concentration in 50 c.c. as he had in 10 c.c. in his rocker tube method.

** Rice II of Miller. Same concentration as Rice I.

*** Yeast counts on triplicate flasks containing the stimulants.

**** Add 0.2 for check.

***** Milligrams of dried alfalfa extract per 100 c.c. of media.
**TABLE XXXIV**

Yeast Counts for Bios I and II Prepared from Alfalfa Fraction III and IV. Concentration Equals Five Times Miller's Preparation. Concentration in 60 c.c. of Yeast Media Same as Miller's.

**Bios I and II from Alfalfa Fraction III**

<table>
<thead>
<tr>
<th>Alfalfa Bios</th>
<th>Miller's Bios</th>
<th>Yeast Counts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 c.c.</td>
<td></td>
<td>22</td>
</tr>
<tr>
<td>1 c.c.</td>
<td></td>
<td>28</td>
</tr>
<tr>
<td>2 c.c.</td>
<td></td>
<td>37</td>
</tr>
<tr>
<td>2 c.c.</td>
<td></td>
<td>41</td>
</tr>
<tr>
<td>3 c.c.</td>
<td></td>
<td>39</td>
</tr>
<tr>
<td>3 c.c.</td>
<td></td>
<td>39</td>
</tr>
<tr>
<td>1 c.c.</td>
<td></td>
<td>53</td>
</tr>
<tr>
<td>1 c.c.</td>
<td></td>
<td>53</td>
</tr>
<tr>
<td>2 c.c.</td>
<td></td>
<td>55</td>
</tr>
<tr>
<td>2 c.c.</td>
<td></td>
<td>53</td>
</tr>
<tr>
<td>1 c.c.</td>
<td></td>
<td>103</td>
</tr>
<tr>
<td>1 c.c.</td>
<td></td>
<td>103</td>
</tr>
<tr>
<td>2 c.c.</td>
<td></td>
<td>103</td>
</tr>
<tr>
<td>2 c.c.</td>
<td></td>
<td>103</td>
</tr>
</tbody>
</table>

**Bios I and II Prepared from Alfalfa Fraction IV (same concentration as for previous fraction)**

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1 c.c.</td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>2 c.c.</td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>3 c.c.</td>
<td></td>
<td>7</td>
</tr>
<tr>
<td>4 c.c.</td>
<td></td>
<td>8</td>
</tr>
</tbody>
</table>
**TABLE XXXIV.** (Continued)

<table>
<thead>
<tr>
<th>Rios I</th>
<th>Rios II</th>
<th>Rios I</th>
<th>Rios II</th>
<th>Heart Counts</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 c.c.</td>
<td>1 c.c.</td>
<td>2 c.c.</td>
<td>1 c.c.</td>
<td>36</td>
</tr>
<tr>
<td>3 c.c.</td>
<td>3 c.c.</td>
<td>4 c.c.</td>
<td>4 c.c.</td>
<td>39</td>
</tr>
<tr>
<td>4 c.c.</td>
<td>1 c.c.</td>
<td>3 c.c.</td>
<td>2 c.c.</td>
<td>43</td>
</tr>
<tr>
<td>2 c.c.</td>
<td>3 c.c.</td>
<td>1 c.c.</td>
<td>4 c.c.</td>
<td>39</td>
</tr>
<tr>
<td>3 c.c.</td>
<td>2 c.c.</td>
<td>1 c.c.</td>
<td>4 c.c.</td>
<td>45</td>
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</tbody>
</table>

Check Counts on Medium 3:

<p>| | | | |</p>
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</thead>
<tbody>
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<td>19</td>
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<td>23</td>
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<tr>
<td>24</td>
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<td></td>
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</tbody>
</table>
# TABLE XXXV

An Attempted Fractionation of the Proteins of Aspergillus niger

into Bic I and II

<table>
<thead>
<tr>
<th>Bic I from Bic II</th>
<th>Bic II from Bic I</th>
<th>Bic I from Bic II</th>
<th>Bic II from Bic I</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 c.c.</td>
<td>0.5 c.c.</td>
<td>0.5 c.c.</td>
<td>0.5 c.c.</td>
</tr>
<tr>
<td>0.5 c.c.</td>
<td>0.5 c.c.</td>
<td>0.5 c.c.</td>
<td>0.5 c.c.</td>
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<tr>
<td>1.0 c.c.</td>
<td>1.0 c.c.</td>
<td>1.0 c.c.</td>
<td>1.0 c.c.</td>
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<tr>
<td>1.0 c.c.</td>
<td>1.0 c.c.</td>
<td>1.0 c.c.</td>
<td>1.0 c.c.</td>
</tr>
<tr>
<td>2.0 c.c.</td>
<td>2.0 c.c.</td>
<td>2.0 c.c.</td>
<td>2.0 c.c.</td>
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<tr>
<td>3.0 c.c.</td>
<td>3.0 c.c.</td>
<td>3.0 c.c.</td>
<td>3.0 c.c.</td>
</tr>
<tr>
<td>4.0 c.c.</td>
<td>4.0 c.c.</td>
<td>4.0 c.c.</td>
<td>4.0 c.c.</td>
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<tr>
<td></td>
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<td></td>
</tr>
<tr>
<td>0.5 c.c.</td>
<td>0.5 c.c.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 c.c.</td>
<td>0.5 c.c.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.0 c.c.</td>
<td>1.0 c.c.</td>
<td></td>
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</tr>
<tr>
<td>1.0 c.c.</td>
<td>1.0 c.c.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.0 c.c.</td>
<td>2.0 c.c.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.0 c.c.</td>
<td>3.0 c.c.</td>
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</tr>
<tr>
<td>4.0 c.c.</td>
<td>4.0 c.c.</td>
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</tr>
</tbody>
</table>

Check Medium 3  
76 - 35 - 35
A Comparison of the Growth Curves of the Various Stimulants

According to Buchanan (1930) and Buchanan and Pelzer (1938) the life history of a typical yeast begins with a period of quiescence, budding then sets in slowly at first but gradually more rapidly until "the stage of logarithmic growth" is reached. After this period, the rate of reproduction decreases and in the last stage there is negative growth acceleration, or more cells die than are formed. Miller (1930) lays great stress on the value of growth curves in estimating and studying Rice preparations. He says in part:

"The slope of the logarithmic curve and the crop obtained when logarithmic production ceases are the important factors; the former is characteristic for a given preparation, the latter measures the amount of active substance present, or if there be more than one that of the active substance present in (physiologically) the least amount. If a suitable race of yeast be used, the crop increases so slowly that the logarithmic stage is passed that a few hours delay in measuring this is of no practical importance."

Comparative growth curves were run on the various fractions of Rice obtained. The material being tested was added to medium C in the desired ratio and the flasks after incubation placed in a rocker in an incubator, (Clarke, E. A., 1923). This furnished sufficient agitation to enable one to obtain accurate and consistent results. These flasks were removed from time to time in order that counts might be taken.

It is interesting to note how well the growth curves (Graph No. III) tally with Miller's predictions. Curves I and II are for the same material but in different amounts, the two curves are nearly parallel showing the common properties of the stimulant while the crop obtained when logarithmic growth ceases
in larger in the case of the larger quantity of stimulant. The
curves for Rios I and II are of considerably different elevation
for the same stimulant indicating a different action.

### Table XXXI

<table>
<thead>
<tr>
<th>Water Content, Low Elvina</th>
<th>Water Content, Low Elvina</th>
<th>Water Content, Low Elvina</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.010</td>
<td>1.020</td>
</tr>
<tr>
<td>1</td>
<td>0.401</td>
<td>0.401</td>
</tr>
<tr>
<td>2</td>
<td>0.771</td>
<td>0.771</td>
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<tr>
<td>3</td>
<td>1.170</td>
<td>1.170</td>
</tr>
<tr>
<td>4</td>
<td>1.570</td>
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<tr>
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<tr>
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<td>7</td>
<td>2.770</td>
<td>2.770</td>
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<tr>
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<td>7.970</td>
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### Table XXXII

<table>
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<th>Water Content, Low Elvina</th>
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</thead>
<tbody>
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<tr>
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<td>0.401</td>
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<td>2500</td>
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<tr>
<td>4</td>
<td>1000</td>
<td>5000</td>
</tr>
</tbody>
</table>

1. Use sodium citrate as control.

2. A = Alcohol other soluble fraction from amygdalin eluate on glycine, 20 mg. per 100 cc.

3. B = Methanol other soluble fraction from amygdalin eluate on glycine, 100 mg. per 100 cc.

4. C = Mio H - 2 cc., insoluble - 20 mg. per 100 cc.

5. D = Mio H alone - 2 cc. per 100 cc. of buffer.


7. F = Mio H - 6 cc., Mio I - 6 cc. per 100 cc.

Graph I
Comparison of growth curves for various stimulants at different concentrations of mold stimulant. Data from Page 73.

Graph II
Comparison of growth curves for various fractions. Data Page 73-74.
SUMMARY

1. It has been shown that the fungi Aspergillus niger, 
   Aspergillus clavatus, Trichoderma linum, Rhizopus 
   nigricans, and Aspergillus terreus are capable of 
   producing a substance or substances stimulating 
   yeast growth.

2. It has been shown that this material of a Bies nature 
   is produced from both glycerine and sucrose substrates.

3. The Bies producing properties of Aspergillus niger have 
   been studied with respect to the composition of 
   the media, production of acids, and of mycelium.

4. A study of the nature of the stimulant has been made.

5. It has been shown that the Bies produced by acid is 
   apparently not the same as Miller's Bies fractions.

6. A synthetic medium has been devised which gives high 
   yields of acid and mycelium with Aspergillus niger.


French:

Rauvin, (1905), J. Etudes cliniques sur la vegetation des

Schoppeyer and Kulzer, (1931), Production of Yeast Growth

Spratling, R. H., (1926), Fractionation of Bic. III. Yeast.
Ind. Eng. Chem. (9): 339-341

Svensk, N., (1929), Studies on Bic. Part VI and VII.


Tobias, R. D., (1936), Studies in the Physiology of the Yeast.
V. Formation of the Species of Certain Yeast in Relation to Salt Concentration.
Ind. Eng. Chem. 28: 261-290

and Fermentations. Sage Ltd.
The Chapman Co.

Williams, M., (1961), Nouvelle Substance indispensale

Williams, G. R., and McAvoy, P. E., The Further Fractionation
of Yeast Bic. Acta, and Their Relationship to