Prevalence and Detection of Lipolytic Microorganisms in Soybean Seeds

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ABSTRACT

In at least one of several *in vitro* tests made, virtually all microorganisms isolated from various lots of stored soybean seeds indicated an ability to alter fatty substrates. This ability of a given microorganism could be dramatically affected by any of several factors, including competition from near-by colonies, submersion in the agar medium, and constitution of the growth medium and (or) choice of indicators used for detection of lipolytic activity. The addition of sodium chloride, useful for adjusting osmotic concentration to allow growth of important xerophytic fungi invading soybean seeds at certain moisture-temperature levels, could also change ability of organisms to produce a positive test for lipolysis. It was not possible to "screen" for lipolytic microorganisms by use of any single method.

A simple, rapid, and precise method of determining lipolytic potential of bacteria *in vitro* has been the subject of several investigations, and the majority of methods developed so far prescribe media containing various dyes in the presence of an emulsion of some fatty substance. A change in color of the dye near growing colonies indicates lipolytic activity. Toxic properties of these dyes, at concentrations needed for proper detection of lipase action, have been one difficulty in their use. Recently a promising method was developed by Sierra (1), who incorporated esters of polyoxyalkylene derivative of sorbitan (Tween) into media; lipolysis was indicated when readily visible crystals (presumably calcium salts) formed around colonies growing on media containing these compounds. He pointed out several advantages of this method, including lack of toxicity.

The present study was initiated to determine the prevalence, in soybean seeds, of microflora capable of producing those enzymes normally associated with alteration of fatty substrates. An attempt was made to evaluate some of the most commonly used methods of detecting lipolytic ability of microorganisms for their suitability as a detector of these microorganisms in soybean seeds. Since some fungi invading seeds during storage may require a substrate of high osmotic pressure and grow sparsely or not at all on many ordinary substrates used in the laboratory, there was a need for detecting what effect, if any, osmotic concentration of the plating medium may have on detection or production of lipase, or both.

MATERIALS AND METHODS

Soybean oil, dyes, or Tweens were incorporated into one of the following six media containing 2% agar: beef extract (0.3%)-peptone (0.5%); beef extract-peptone plus 5% NaCl; Czapek-Dox plus 30 or 50% sucrose; V-8 juice (Campbell Soup Co.), 200 ml./liter; Peptone salt (1 or 2% peptone plus 5% NaCl); or malt-salt (1% malt extract and 5% NaCl). Fatty substances were either 1% emulsion of Alkalyte soybean oil, 1% Tween-80, or 1% Tween-20, and indicator dyes included 0.01–0.001% malachite green, nile blue, or night blue. Dyes were used with oil, not with Tween.

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Microorganisms were obtained from soybean seeds which had been collected in the field and stored at various relative humidities at room temperatures. Fungi, bacteria, and yeasts were grown from samples either by placing whole surface-sterilized seeds (0.5% sodium hypochlorite for 2 min., followed by two rinses in sterile water) on agar surfaces or in dilution plates after seeds were chopped in a Waring Blendor for 2 or 5 min. in a 0.1% agar solution. Cultures were incubated at 27°C. and were evaluated after 3 to 30 days, depending on growth rates of the organisms involved.

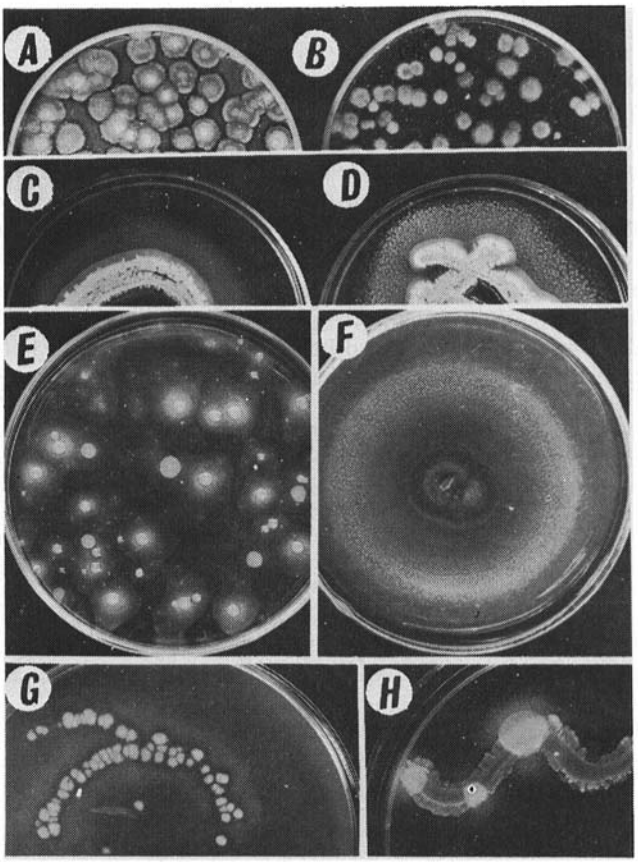

Fig. 1. Lipolytic reaction of microorganisms on beef extract (0.3%)-peptone (0.5%) agar plus 1% glucose and 1% Tween. A, B, lipolytic molds on agar, respectively with and without Tween-20; C, D, lipolytic yeast on agar containing respectively Tween-20 and Tween-80. E, mixed bacteria in agar dilution plate containing Tween-20 (note differences in reaction and that submerged colonies are not lipolytic; crystals formed beyond halos and only on surface of agar). F, mold on agar plus Tween-20 (note indications of lipolytic activity reaching considerably beyond advancing hyphae). G, yeast streaked on agar containing Tween-20; crystals formed beyond halos and only on surface of agar. H, mixed lipolytic and nonlipolytic microorganisms streaked together on agar plus Tween-20; little or no interference of either growth or lipolytic ability was noted here, but it was frequent in some other combinations.

RESULTS AND DISCUSSION

In media containing soybean oil, dyes tended to be toxic and to select out various taxonomic groups of microorganisms, depending on concentration and the dye used. More organisms produced a positive reaction on Czapek-Dox agar plus 30% sucrose and 0.01% night blue than on other media containing night blue. Malachite green gave a very striking reaction with certain lipolytic-positive microorganisms at 0.01% but was toxic to others, and isolations made with agar containing more than about 0.001% of this compound selected out only a portion of the population of microorganisms that could be detected from the same sample when other media were used. Nile blue A was not appreciably toxic, but the pinkish color indicating lipolysis was often faint, indistinct, and difficult to detect among fast-growing fungi.

No toxicity was evident where Tween (with no dye or oil) was used, and it was more convenient to use than oil emulsions. However, assay for percentage of lipolytic microorganisms when Tween was used was not without complications, and some of these are illustrated in Fig. 1: 1) not all organisms produce lipase at the same time or rate; 2) submerged colonies often did not product lipase or produced it more slowly than surface colonies; 3) lipolytic capability of various taxonomic types varied tremendously with the base medium used; 4) lipolytic ability of individual organisms in mixed cultures could be altered by organisms growing near them; and 5) Tween often stimulated growth. Furthermore, the choice of any specific basal medium in which to incorporate Tween for detecting lipolytic ability was difficult. Tables I and II illustrate the fact that often an organism grown in the

TABLE I. EFFECT OF SODIUM CHLORIDE ON LIPOLYTIC ABILITY OF FUNGI ON BEEF EXTRACT PEPTONE AGAR PLUS TWEEN-20

Fungus	Isolate	Maximum Detectable Extension of Lipase-Containing Zone beyond Advancing Hyphae	
		5% Salt mm.	No Salt mm.
Alternaria sp.	1	6	0
Aspergillus ochraceous	1	0	8
	2	5	4
	3	9	0[a]
A. repens	1	0	0
	2	0[a]	0
	3	3	0[a]
	4	0[a]	0
A. ruber	1	0[a]	0
Penicillium spp.	1	0[a]	0[a]
	2	6	4
Unidentified	1	7	5
	2	0[a]	0
	3	11	0[a]
	4	0[a]	0
	5	0	0

[a]Indicated crystal formation under the colony but not extending beyond tips of advancing hyphae.

center of an agar plate could be classified as lipolytic or nonlipolytic, depending on the basal medium, the dye, or the method used. Salt, for example, is used extensively in various media for selection of xerophytic molds that invade seeds during storage; it can have a dramatic effect, not only upon growth of such organisms but apparently upon their lipolytic activity as well. Thus it was not possible to assay a sample simply by plating it out and observing the response of mixed groups of microorganisms emerging, especially on a single base medium. Certain organisms were lipolytic on any of the various media containing Tween, and it was unusual to find an organism that could not produce lipase on at least one of the various media tested.

TABLE II. LIPOLYTIC REACTION OF MICROORGANISMS ISOLATED FROM STORED SEED AND TESTED ON THREE MEDIA

Type of Microorganism	Number of Isolates Tested	Number of Isolates Showing Lipolytic Activity		
		beef-peptone, soybean oil, night blue	beef-peptone, soybean oil, nile blue	beef-peptone, Tween-80
Bacteria	9	5	3	6
Yeasts	5	2	1	1
Storage fungi[a]	23	21	13	22
Field fungi[b]	9	8	8	9

[a] Species in the genera Aspergillus and Penicillium.
[b] Species of *Alternaria, Fusarium, Rhizopus,* and *Helminthosporium.*

It appears that a very high percentage of the microorganisms found associated with soybean seeds during storage show a potential for lipase production *in vitro*. In addition to substantiating findings of other investigators on the importance of base substrate in the determination of lipolysis with certain dyes (2,3), this study indicates that interaction of microorganisms with Tween is also dramatically affected by the medium. Furthermore, microorganisms may influence lipolytic abilities of each other when growing close together on a common medium, and lipolytic surface-growing colonies may not produce lipase when submerged. It thus would appear difficult to "screen" for lipolytic microorganisms by any one of these methods alone. It is furthermore suggested that results of such tests may bear no relation to that which occurs *in vivo*.

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