

Isolation and Identification of Lipolytic Microorganisms Found on Rough Rice from Two Growing Areas

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ABSTRACT

Rice bran and whole brown rice are currently underutilized because free fatty acids are formed from rice oil during storage. Rough rice from two growing areas was tested for presence of lipolytic microorganisms that could release these fatty acids. Approximately 10% of the total bacterial plate count (4×10^6 /g for Louisiana and 12×10^6 /g for the Arkansas rice samples) were lipolytic. Upon testing, most were classified as nonsaccharolytic, alkali-producing pseudomonads. The average mold plate count for the Louisiana sample was 2×10^3 colony forming units (cfu)/g and 5×10^3 cfu/g for the Arkansas sample. All molds isolated showed various amounts of lipolytic action, as determined by the size of the lipolytic zone. The molds were generally isolated more from the Louisiana than from the Arkansas rice. *Alternaria* and *Helminthosporium* species, the most prevalent molds, were found in all samples.

Rice is one of the most important field crops in the world and is the basic food for over one-half of the world's population (6). It is well known that brown rice is more nutritious than polished rice (8). Rice bran is an excellent source of B and E vitamins, minerals, and protein, and is higher in protein, fat, fiber, ash, and vitamins than the starchy endosperm (8). The bran layer and embryo contain most of the lipids (8). Rice bran is underutilized, in view of the quality and quantity of nutrients present (7).

Brown rice has a short shelf life because of decomposition of lipids into fatty acids. This decomposition results not only in free acids but also in a bad taste that reduces the marketing potential of brown rice. There are two theories that describe the cause of lipid decomposition. The first holds that the rice grain itself contains lipolytic enzymes that break down the lipids. The other holds that microorganisms are present that decompose the lipids while the product is in storage and on the store shelf. If the major cause of lipolysis could be defined, it should be possible to retard or decrease lipid decomposition, thereby increasing shelf life and market quality of a valuable foodstuff. The object of this work was to show the presence, identification, and numbers of lipolytic bacteria and molds on rough rice that may influence shelf-life of brown rice.

MATERIALS AND METHODS

Rough rice with intact hulls, Starbonnet variety, was obtained from the Louisiana State Experiment Station, Crowley, La., and the USDA Field Experiment Station, Stuttgart, Ark. The moisture content was reduced by mechanical drying to 9-11% after threshing. Samples were kept in sterile plastic bags and stored at 4 C until needed.

Microbiological examination: Bacteria

Five samplings were made of rice from each location. Ten grams of clean, rough rice was weighed aseptically into a sterile Osterizer jar, 90 ml of sterile .02 M phosphate-buffered distilled water, pH 7.2, was added, and the mixture was blended for 1 min. Serial dilutions of 10^{-4} , 10^{-5} , and 10^{-6} were made in sterile, phosphate-buffered distilled water. Triplicate nutrient agar (BBL) pour plates (total plate count) and tributyrin-based agar plates for isolation of lipolytic bacteria (4) were inoculated with the appropriate dilutions and incubated at 30 C for 3 days.

Lipolytic bacterial colonies displayed a clear zone, indicating lipase activity on the tributyrin-base medium. These colonies were picked and inoculated on Triple Sugar Iron (BBL) Agar slants and incubated for 24 h at 30 C.

Biochemical media

The following biochemical tests were used to identify the isolated lipolytic bacteria (3,5). Gram stains were made from 24-h-old colonies grown on nutrient agar. These colonies were examined for oxidase and catalase production. The carbohydrates tested were in a 0.7% concentration in oxidative-fermentative medium (BBL) as follows: glucose in both open and sealed tubes, and sucrose, xylose, galactose, lactose, maltose, fructose in open tubes only. The tubes containing glucose that were used for fermentation testing were sealed with 3% agar. Although 3% agar does not prevent the egress of oxygen as well as petrolatum does, it was felt that it would retard oxygen diffusion sufficiently during incubation to obtain reliable results. In addition, 3% agar is easier to use. Other tests were citrate, esculin, galactosidase (ONPG), starch, lecithinase, growth on MacConkey agar (Difco), indole, Methyl Red-Voges Proskauer, urea, pigment, motility, flagellar morphology, and reduction of nitrate. After inoculation, media were incubated for 24-48 h at 30 C.

Fungi (molds)

Five samplings were made of rice from each location to survey the mold population. Serial dilutions of rice samples in triplicate (10^{-1} to 10^{-3}) were plated on acidified (pH 3.5 with sterile tartaric acid) potato dextrose agar (PDA; Difco) and incubated at 30 C for 3-5 days. Total mold counts were obtained from the appropriate dilution plates, and representative colonies were isolated on PDA slants.

Each isolate was inoculated on tributyrin-based agar and incubated at 30 C for 5 days. All isolates that exhibited lipolytic activity, as did bacteria, were held for identification.

Lipolytic isolates were cultured by the slide culture method of Riddell (11) except for the *Penicillium* and *Aspergillus* species, which were inoculated (tri-point inoculation) on Czapek's solution agar (Difco) and malt extract agar (10). However, with zygomycetes, only direct microscopic slides were prepared for positive identification. Amann's solution (10) was used to prepare microscopic slides from all cultures.

Microscopic and colonial morphology were employed to determine the taxonomic identity of the fungi (1, 2, 9, 10).

RESULTS AND DISCUSSION

Data in Table 1 show that the rough rice used in these studies has a relatively low population of microorgan-

TABLE 1. *Microbial quality of clean rough rice*

Type of count	Average (No/g)	Range (No/g)
Louisiana Rice		
Total plate count	3.5×10^6	2.2- 4.1×10^6
Lipolytic plate count	6.1×10^5	3.3- 8.3×10^5
Molds	2×10^3	1.8- 2.9×10^3
Arkansas Rice		
Total plate count	12×10^6	9.7- 13.5×10^6
Lipolytic plate count	9.1×10^5	6.5- 12.0×10^5
Molds	5×10^3	1.0- 6.0×10^3

isms. Our study showed that Louisiana and Arkansas rice had similar bacterial populations. In 1970, Goel et al. (6) reported plate counts of raw, wild rice which exceeded 10^9 bacteria per gram, whereas our studies on cultivated rice showed only 10^6 per gram. The lipolytic bacterial count in the present study was 10^5 per gram, or approximately 10% of the total bacterial population. Clear zones of lipolysis were developed in 48 h by the lipolytic bacteria, which showed the presence on rough rice of a considerable population of bacteria capable of breaking bran lipids into fatty acids, thereby affecting taste and shortening the shelf life of brown rice. The majority of the lipolytic bacteria were gram negative rods unable to ferment glucose and were classified as alkali-producing pseudomonads. The identity of the lipolytic bacteria in the Louisiana and Arkansas rice was similar, as can be seen in Table 2. Most of these isolates

TABLE 2. *Lipolytic bacteria isolated from Louisiana and Arkansas rice samples at 30 C for 72 hours*

Louisiana		Arkansas	
Isolate	Number isolated	Isolate	Number isolated
<i>Pseudomonas alcaligenes</i>	115	<i>Pseudomonas alcaligenes</i>	142
<i>P. diminuta</i>	22	<i>P. cepacia</i>	12
<i>P. putrefaciens</i>	2	<i>P. pseudoalcaligenes</i>	3
<i>P. cepacia</i>	1	<i>P. aeruginosa</i>	1
<i>Xanthomonas</i> sp.	27	<i>Xanthomonas</i> sp.	6
<i>Flavobacterium</i> sp.	13	<i>Enterobacter agglomerans</i>	2
<i>Chromobacterium typhiflavum</i>	8	<i>Chromobacterium typhiflavum</i>	2
<i>Proteus mirabilis</i>	1	<i>Micrococcus luteus</i>	1
<i>Micrococcus varians</i>	1		
<i>Staphylococcus saprophyticus</i>	10		
<i>Corynebacterium</i> sp.	1		

were identified as *Pseudomonas alcaligenes*, followed in number by other nonsaccharolytic alkali-producing pseudomonads and xanthomonads. Also present were some saccharolytic gram-negative bacteria such as *Flavobacterium* species, and a few that were classified as *Chromobacterium typhiflavum*. Two isolates of *Entero-*

bacter agglomerans were found in Arkansas rice whereas in Louisiana rice a lipolytic isolate identified as *Proteus mirabilis* was found. Few gram-positive lipolytic bacteria were isolated from either sample. All but one belonged to the family *Micrococcaceae*, these being *Micrococcus luteus* in the Arkansas rice and *Staphylococcus saprophyticus* and *Micrococcus varians* in the Louisiana rice. An isolate identified as a *Corynebacterium* sp. was also found in the Louisiana sample. Although individual counts and isolates of the two rice samples were different, the data show that the predominant lipolytic bacteria present on rough rice were nonsaccharolytic, alkali-producing pseudomonads.

Mold count data (Table 1) show that the number of molds in the Arkansas rice was slightly higher than that from the Louisiana, although all were in the range of 1,000 to 6,000 colony-forming units (cfu) per gram of rice.

In Table 3 the filamentous fungi isolated for the two locations are identified. Louisiana rice yielded more

TABLE 3. *Taxonomic identification of lipolytic molds^a*

LOUISIANA SAMPLE	ARKANSAS SAMPLE
<i>Alternaria</i> sp.	<i>Helminthosporium</i> sp.
<i>Helminthosporium</i> sp.	<i>Alternaria</i> sp.
<i>Curvularia</i> sp.	<i>Penicillium</i> sp.
<i>Penicillium</i> sp. ^b	<i>Curvularia</i> sp.
<i>Aspergillus flavus</i>	Unidentified organism
Unidentified organism	<i>Rhizopus</i> sp.
<i>Cladosporium</i> sp.	
<i>Scopulariopsis</i> sp.	
<i>Paecilomyces</i> sp.	
<i>Mucor</i> sp.	
<i>Rhizopus</i> sp.	
<i>Fusarium</i> sp.	

^aListed in order of prevalence from the top.

^bRepresents *P. chrysogenum*, *P. oxalicum* series, and unidentified *Penicillium* species.

mold genera than did the samples from Arkansas. *Alternaria* and *Helminthosporium* species were much more prevalent in both locations than any of the other genera, although specific numbers of each genus were not determined. These "field molds" have been associated with rice in other studies (12, 13). *Fusarium* sp., on the other hand, were infrequently isolated from Louisiana samples, and none were found in the Arkansas samples. This organism is considered a common member of rice mycoflora.

Both *Aspergillus* and *Penicillium* species were found in low numbers in samples from Louisiana. *Aspergillus flavus* and *Penicillium chrysogenum* were the principal species found; the former was not isolated from any of the Arkansas samples.

One other lipolytic mold (labeled unidentified organism) was commonly isolated in low numbers. Initially, it produced red pigmentation in the mycelium and in the agar which turned dark with age. Since conidial structures were not observed, its taxonomy was not established.

Although the moisture content of rough rice after drying is between 9-11%, too low for microbial activity,

lipolysis is a problem during prolonged storage of whole brown rice. If brown rice is stored in large containers, such as bags or barrels, heat plus humidity can raise the moisture level to a point where microbial activity could begin. For long periods of storage, whether in a warehouse or on a market shelf, microbial lipases could act on the rice, particularly if the rice is not vacuum packed. This study has shown that both lipolytic fungi and bacteria are present in sufficient numbers to cause rancidity and off-flavor production under appropriate conditions during storage.

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