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Microbiology of pollen and bee bread : taxonomy and enzymology of molds*

M. Gilliam, D. B. Prest and B. J. Lorenz

US Department of Agriculture, Agricultural Research Service, Carl Hayden Bee Research Center, 2000 E. Allen Road, Tucson, AZ 85719, USA

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Summary — One-hundred and forty-eight molds were isolated from the following samples of almond, *Prunus dulcis,* pollen : floral pollen collected by hand; corbicular pollen from pollen traps placed on colonies of honey bees, *Apis mellifera,* in the almond orchard; and bee bread stored in comb cells for one, three, and six weeks. The majority of molds identified were Penicillia (32%), Mucorales (21%), and Aspergilli (17%). In general, the number of isolates decreased in pollen as it was collected and stored by the bees. Each type of pollen sample appeared to differ in regard to mold flora and dominant species. *Aureobasidium pullulans, Penicillium corylophilum, Penicillium crustosum,* and *Rhizopus nigricans* were among the molds that may have been introduced by bees during collection and storage of pollen. *Mucor* sp., the dominant mold in floral pollen, was not found in corbicular pollen and bee bread. Tests for 19 enzymes revealed that most of the molds produced caprylate esterase-lipase, leucine aminopeptidase, acid phosphatase, phosphoamidase, ß-glucosidase, and *N*-acetyl-ß-glucosaminidase. Thus, enzymes involved in lipid, protein and carbohydrate metabolism were produced by pollen molds. Molds could also contribute organic acids, antibiotics and other metabolites.

pollen — bee bread — molds

Résumé — Microbiologie du pollen et du pain d'abeilles : taxonomie et enzymologie des moisissures. *A l'aide de divers milieux microbiologiques possédant des pH différents, on a isolé 148 moisissures des échantillons suivants de pollen d'amandier,* Prunus dulcis *: pollen de fleurs récolté à la main; pollen en pelotes prélevé dans les trappes à pollen posées sur des colonies d'abeilles (*Apis mellifica*) dans un verger d'amandiers; et pain d'abeilles stocké dans les cellules des rayons durant une, 3 et 6 semaines. La majorité des moisissures identifiées sont des Penicillia (32%), des Mucorales (21%) et des Aspergillia (17%). C'est le pollen de fleurs qui est le plus riche en isolats, mais le plus pauvre en espèces. En général le nombre d'isolats diminue dans le pollen quand il est récolté et stocké par les abeilles. Chaque type d'échantillon pollinique semble différer des autres par la flore de moisissures et les espèces dominantes. Puisque les moisissures sont identifiées d'après les besoins de croissance et la caractérisation microscopique et macroscopique des structures morphologiques, les données biochimiques ne proviennent pas des tests taxonomiques. On a*

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donc analysé 19 enzymes chez 78 isolats, représentant 28 espèces, par le système API ZYM. Aucune moisissure ne produit de trypsine, de ß-glucuronidase, ni de α-mannosidase. La plupart des moisissures produisent de la caprylate lipase-estérase, de la leucine aminopeptidase, de la phosphatase acide, de la phosphoamidase, de la ß-glucosidase et de la N-*acétyle-ß-glucosaminidase. Les moisissures du pollen produisent donc des enzymes impliqués dans le métabolisme des protéines, des lipides et des glucides.*

Ces résultats suggèrent que la flore de moisissures du pollen en pelotes et du pain d'abeilles peut résulter d'inoculations microbiennes par les abeilles et de modifications chimiques du pollen dues aux substances ajoutées par les abeilles lors de la régurgitation du contenu du jabot et à la fermentation microbienne, qui permet à certaines espèces de survivre et à d'autres pas. Même si, dans nos échantillons, les moisissures étaient plus nombreuses que les espèces de Bacillus *et les levures, le pollen est rarement envahi par elles. Parce qu'elles sont susceptibles de fournir des enzymes, des acides organiques, des antibiotiques et d'autres métabolites, les moisissures méritent des études plus approfondies.*

pollen — pain d'abeilles — moisissures

Zusammenfassung — Mikrobiologie von Pollen und Bienenbrot : Taxonomie und Enzymologie des Schimmels. *Unter Verwendung verschiedener mikrobiologischer Medien mit unterschiedlichem pH-Wert wurden 148 Schimmelpilze von den folgenden Proben von Mandelpollen (*Prunus dulcis*) untersucht : Blütenpollen (von Hand gesammelt), Pollenhöschen (aus Pollenfallen an Bienenvölkern* [Apis mellifera] *im Mandelbaumgarten) und Bienenbrot, das 1, 3 und 6 Wochen in der Wabe gespeichert war.*

Die am häufigsten auftretenden Schimmelpilze waren Penicillia (32%), Mucorales (21%) und Aspergilli (17%). Der Blütenpollen lieferte die meisten Isolate, aber die wenigsten Arten. Im allgemeinen nahm die Anzahl der Isolate im Pollen mit dem Sammeln und Speichern durch die Biene ab. Jeder Pollentyp schien im Hinblick auf die Schimmelflora und die dominierenden Arten verschieden zu sein.

Da die Schimmelpilze aufgrund ihrer Wachstumserfordernisse sowie mikroskopischer wie makroskopischer Charakterisierung von morphologischen Strukturen identifiziert werden, erhält man von taxonomischen Untersuchungen keine biochemischen Daten. Daher wurden 78 Isolate, die 28 Arten repräsentieren, mit dem API ZYM-System auf 19 Enzyme analysiert. Kein Schimmelpilze produzierte Trypsin, ß-Glucuronidase oder α-Mannosidase. Die meisten Schimmelpilze produzierten Caprylat-Esterase-Lipase, Leucin-Aminopeptidase, saure Phosphatase, Phosphoamidase, ß-Glucosidase und N-*Acetyl-ß-Glucosaminidase. Dies bedeutet, daß die untersuchten Pollenschimmel Enzyme des Protein-, Fett- und Kohlehydratstoffwechsels produzieren.*

Diese Ergebnisse deuten darauf hin, daß die Schimmelflora im Höselpollen und im Bienenbrot ein Ergebnis folgender Einflüsse ist : mikrobielle Inokulation und chemische Veränderung des Pollens durch Zugabe von Honigmageninhalt durch die Biene; Drüsensekretion sowie mikrobielle Fermentation, die manche Schimmelarten tolerieren, andere nicht. Obwohl Schimmelpilze in unseren Proben weit zahlreicher waren als Bacillus *spp. oder Hefen, wurde der Pollen selten von Schimmel überwachsen. Die Schimmelpilze sollten als potentielle Spender von Enzymen, organischen Säuren, Antibiotika und anderer Metabolite intensiver untersucht werden.*

Pollen — Bienenbrot — Schimmel

Introduction

Studies have shown for many years that pollen and bee bread, that is pollen stored in comb cells of the hive, differ biochemically, and extensive analyses have been conducted on various floral and bee-collected (corbicular) pollens. The conversion of pollen to bee bread has often been postulated to be the result of microbial action, principally a lactic acid fermentation caused by bacteria and yeasts (Foote, 1957; Haydak, 1958). However,

the chemical and biochemical changes occurring in pollen as it is collected and stored by honey bees, *Apis mellifera,* are not clearly understood, and relatively little is known about the microbiology of pollen and bee bread.

To better understand the nutrition of honey bees, we studied the chemical, biochemical, and microbiological composition of pollen from a single plant species before, during, and after storage in comb cells. Previous papers on the subject by researchers at the Carl Hayden Bee Research Center reviewed earlier work and reported results concerning yeasts (Gilliam, 1979a); *Bacillus* ssp. (Gilliam, 1979b); fatty acids, sterols, vitamins, titratable acidity, minerals (Loper *et al.,* 1980); and protein content, amino acids, selected enzymes, pH, and 10-hydroxy-Δ^2-decanoic acid (Standifer *et al.,* 1980). In all this work, the same samples of almond, *Prunus dulcis (Prunus communis),* pollen were utilized.

Even though molds are widely known for their abilities to degrade and to synthesize numerous compounds including the production of many materials important to the drug, food and chemical industries, they have received scant attention in apicultural research concerned with pollen and bee bread. Early mycological research recognized that certain molds are common saprophytes on and inside honey bees and brood combs, but efforts were concentrated on dead bees; combs, particularly from dead colonies; and moldy pollen (Betts, 1912; Burnside, 1927). Betts (1912) reported a species of *Cladosporium* as well as *Mucor erectus* in corbicular pollen and *Bettsia alvei, Eremascus fertilis, Gymnoascus setosus, Oospora favorum,* and *Penicillium crustaceum* in pollen stored in combs. She noted that honey appeared to be immune to attacks of molds. Burnside (1927) stated that most of the fungi collected by widespread foraging of honey bees are probably unable to become established within the bee or the hive. He found that Penicillia were the most common molds within the hive, Aspergilli occurred less frequently, and species of *Mucor* did not grow well on brood combs.

Fungus-caused spoilage of provisions and mortality of honey bees are rare (Batra *et al.,* 1973). Recently Gilliam and Vandenberg (1988) reviewed the literature on fungi pathogenic or detrimental to honey bees. Only *Ascosphaera apis* which causes chalkbrood disease in honey bees is of economic importance. The pollen mold, *Bettsia alvei,* is not a serious problem since it does not grow well in cells that are filled with pollen and finished with a layer of honey on top (Skou, 1972).

Burri (1947) stated that pollen is germfree in blossoms that have not opened as well as in opened blossoms if uncontaminated by insect visitation or air currents. Neither of the two microbiological studies of pollen and bee bread (Chevtchik, 1950; Pain and Maugenet, 1966) gave data on molds, although Chevtchik (1950) mentioned *B. alvei as* a possible consumer of lactic acid in bee bread.

Arizan *et al.* (1967) isolated *Absidia ramnosa, Aspergillus flavus, Aspergillus fumigatus, Aspergillus niger, Aspergillus terreus, Aspergillus versicolor, Mucor mucedo, Penicillium clavigerum, Penicillium purpurogenum, Rhizopus nigricans* and *Trichothecium roseum* from Indian corn pollen collected by machine. Sainger *et al.* (1978) reported that *Alternaria alternata* was the most common isolate in pollen from 3 herbaceous annual plants. Other molds isolated were *Aspergillus flavus, Aspergillus luchuensis, Aspergillus nidulans, Aspergillus sulphureus, A. versicolor, Cladosporium oxysporum, Epicoccum purpurascens, Fusarium oxy-*

sporum, Monilia fructigena, Monilia sitophila, Monilia sp., *Rhizopus* sp., *R. nigricans* and *Trichoderma viride.* Molds isolated from bee bread by Egorova (1971) were *A. flavus, A. versicolor, Mucor alboalter, Penicillium granulatum, Penicillium solitum* and *Sporotrichum olivecum.*

The present paper reports the results of the isolation and identification of molds from almond floral pollen, corbicular pollen, and bee bread stored in comb cells; analyses of enzymes produced by selected isolates; and comparison of species isolated with those previously reported from honey bees in Arizona.

Materials and Methods

Details concerning bee colonies and collections of pollen and bee bread are given by Gilliam (1979a), Loper *et al.* (1980), and Standifer *et al.* (1980). The following samples of almond pollen were examined : fresh floral pollen collected by hand; corbicular pollen containing 99.8% almond pollen from pollen traps placed on bee colonies in the almond orchard; and bee bread stored in cells for 1, 3, and 6 weeks in newly drawn combs of colonies of bees maintained in a greenhouse. Each sample was divided into 4 sub-samples of approximately 0.75 g each. Then each of the 4 sub-samples was homogenized by hand in 2.5 ml of sterile 0.85% NaCl in a glass tissue grinder. The homogenates were plated (0.1 ml) in duplicate on acidified yeast extract-malt extract agar containing 1% glucose, pH 3.7—3.8 (Miller *et al.*, 1976); mycological agar with low pH (Difco, pH 4.8); nutrient agar (Difco) acidified with 0.1 N HCl to pH 5.0; and eugon agar (Difco, pH 7.0). One plate from each sub-sample was incubated at 25°C and one at 37°C. All were incubated under aerobic conditions except eugon agar plates which were placed in 4% CO_2. During a 2-week incubation period, plates were examined periodically for mold growth.

When molds appeared, they were transferred to plates of Czapek solution agar or malt extract agar (Difco) to allow time for sporulation and to test for purity. These plates were incubated at 25°C under aerobic conditions. Pure cultures of isolates were lyophilized for preservation until tests were conducted. Molds were tested and identified according to Neergaard (1945), DeVries (1952), Cooke (1959), Ames (1961), Booth (1961), Morton and Smith (1963), Ellis (1965), Raper and Fennell (1965), Raper and Thom (1968), Zycha *et al.* (1969), Kendrick and Carmichael (1973), Samson (1974), von Arx (1975) and McGinnis *et al.* (1986).

Since molds, in contrast to bacteria and yeasts, are identified on the basis of growth requirements and microscopic and macroscopic characterization of morphological structures, biochemical data do not result from tests for identification. Therefore, selected isolates were tested for 19 enzymes with the API ZYM system (Analytab Products) using the methods of Bridge and Hawksworth (1984). Suspensions of some isolates such as *Mucor* ssp. and *Alternaria tenuis* were sonicated for 1—10 min to separate fungal spores before inoculation into the API ZYM strips. Also, malt extract agar and/or potato dextrose agar (Difco) were used to prepare inocula of some cultures when growth appeared to be less than optimal on Czapek solution agar.

Results

No attempt was made to determine whether spores or mycelial elements were isolated from pollen and bee bread. However, molds were isolated on all 4 media used (Table I). Seventy-seven percent of the isolates were from media incubated at 25°C, and 23% were from media at 37°C which is near the brood nest temperature of 34°C (Dunham, 1929). However, the optimum temperatures for most fungi are in the range of 20—30°C (Alexopoulos, 1962). Isolations from floral pollen increased with decreasing pH of the media. In contrast, the highest percent of isolations from corbicular pollen from the pollen trap was on acidified nutrient agar with a pH of 5.0. Few isolations were made from floral or corbicular pollen on eugon agar with a pH of 7.0, but this situation changed with bee bread. The

Table I. Percent of molds isolated on various microbiological media from each pollen source.

Source of pollen	*Media* [a] *(pH)*			
	YMA (3.7-3.8)	*MYC (4.8)*	*NA-Ac (5.0)*	*EA (7.0)*
Flower	37	32	27	5
Pollen trap	20	30	43	7
Comb cells after 1 week	15	36	21	27
Comb cells after 3 weeks	14	33	24	29
Comb cells after 6 weeks	22	30	30	17
All sources combined	23	32	29	16

[a] YMA = acidified yeast extract-malt extract agar with 1% glucose; MYC = mycological agar with low pH; NA-Ac = acidified nutrient agar; EA = eugon agar.

percent of isolations on various media was similar in bee bread samples stored for one week and for 3 weeks. These results indicate that a variety of media with different chemical compositions and pH values incubated at both 25°C and 37°C aerobically and under CO_2 should be used for determining mycoflora of pollen and bee bread.

One-hundred forty-eight molds were isolated from pollen and bee bread, of which 139 were identified (Table II). Overall, the majority of molds identified were Penicillia (32%), Mucorales (21%), and Aspergilli (17%). Floral pollen yielded the highest number of isolates but the smallest number of species. In contrast, bee bread stored in comb cells for 3 weeks had the fewest isolates but the greatest number of different species. In general, the number of isolates decreased in pollen as it was collected and stored by bees.

The most frequent isolate was *Mucor* sp., associated exclusively with floral pollen. All 19 isolates appeared similar, but species identification was not made. They were characterized by the production of coenocytic mycelium, some sympodially branched sporangiophores containing gemmae (chlamydospores) and yellow globules, globose sporangia with finely spinose walls, columella with a distinct collarette, and elliptical smooth sporangiospores. The other molds identified from floral pollen were found in at least one additional pollen source.

The second most frequently isolated mold was *Penicillium corylophilum.* It was associated with corbicular pollen and all bee bread sources but not with floral pollen. This was also the case for R. *nigricans.* Other species which first appeared in corbicular pollen and were then found in bee bread were *Aureobasidium pullulans, Cladosporium herbarum, Penicillium chrysogenum,* and *Penicillium crustosum. Aspergillus niger* was a frequent isolate and was found in all types of pollen samples except bee bread stored for one week and was isolated most often from bee bread stored for 6 weeks. Another frequently encountered mold was *Penicillium cyclopium* which was most abundant in bee bread stored for one week and was found in all sample types except corbicular pollen. *Cladosporium cladosporioides* was the only species isolated from all sample types but was most prevalent in floral pollen. Molds isolated from more

Table II. Molds isolated from almond pollen and bee bread.

Molds	*No. of isolates*
Isolates from floral pollen	
Mucor sp.	19
Alternaria tenuis	6
Cladosporium cladosporioides	5
Aspergillus niger	3
Penicillium brevi-compactum	3
Penicillium cyclopium	2
Unidentified	3
Total	41
Isolates from corbicular pollen	
Penicillium corylophilum	6
Penicillium crustosum	6
Aureobasidium pullulans	3
Rhizopus nigricans	3
Cladosporium cladosporioides	2
Penicillium brevi-compactum	2
Aspergillus niger	1
Aspergillus versicolor	1
Cladosporium herbarum	1
Penicillium chrysogenum	1
Peyronelia sp.	1
Scytalidium sp.	1
Unidentified	2
Total	30
Isolates from bee bread stored in comb cells for 1 week	
Penicillium cyclopium	8
Penicillium corylophilum	7
Rhizopus nigricans	4
Aureobasidium pullulans	4
Alternaria tenuis	2
Penicillium crustosum	2
Arthrinium phaeospermum	1
Aspergillus flavus	1
Cladosporium cladosporioides	1
Cladosporium herbarum	1
Scopulariopsis brevicaulis	1
Unidentified	1
Total	33
Isolates from bee bread stored in comb cells for 3 weeks	
Aspergillus flavus	3
Cladosporium cladosporioides	2
Penicillium chrysogenum	2
Penicillium corylophilum	1
Penicillium janthinellum	1
Aspergillus amstelodami	1
Aspergillus niger	1
Aspergillus ustus	1
Cladosporium sphaerospermum	1

Table II. (continued)

Mucor racemosus	1
Paecilomyces varioti	1
Penicillium cyclopium	1
Rhizopus nigricans	1
Thielavia sepedonium	1
Xylohypha bantiana	1
Unidentified	2
Total	21
Isolates from bee bread stored in comb cells for 6 weeks	
Aspergillus niger	7
Aspergillus flavus	3
Chaetomium elatum	3
Penicillium corylophilum	2
Aspergillus amstelodami	1
Aspergillus flavus var. *columnaris*	1
Chaetomidium pilosum	1
Cladosporium cladosporioides	1
Paecilomyces varioti	1
Penicillium cyclopium	1
Rhizopus nigricans	1
Unidentified Ascomycete	1
Total	23

than one source of bee bread but not floral or corbicular pollen were *Aspergillus amstelodami, A. flavus,* and *Paecilomyces varioti. Alternaria tenuis* was found in floral pollen and bee bread stored for one week, and *Penicillium brevi-compactum* was in floral and corbicular pollen but not bee bread. Other than *Chaetomium elatum* which was found in bee bread stored for 6 weeks, the remaining species that were identified appeared once in only one type of pollen source other than floral pollen. Thus, each type of pollen sample appeared to differ in regard to mold flora and dominant species since the predominant mold in almond floral pollen was *Mucor* sp.; in corbicular pollen, *P. corylophilum* and *P. crustosum* were most common; in bee bread stored in comb cells for one week, *P. cyclopium* and *P. corylophilum* were the most numerous isolates; in bee bread stored for 3 weeks, there was no obvious dominant species; and in bee bread stored for 6 weeks, *A. niger* was most common.

Eight unidentified molds in Table II were non-viable after lyophilization. We were also unable to assign the unidentified Ascomycete to genus. It produced ostiolate dark ascocarps with unbranched terminal hairs. The asci were cylindrical and contained 4 ascospores. Ascospores were ellipsoidal to ovoid, smooth, non-apiculate, and yellow-brown to brown. Since it was difficult to determine by light microscopy whether the ascospores were single or double-pored, scanning electron microscopy was used to reveal that the majority were single-pored, although double-pored ascospores were also present.

To determine enzymatic activity of the molds from pollen and bee bread, attempts were made to test at least one strain of each species and more strains of

the species frequently isolated. However, *Cladosporium sphaerospermum* did not survive lyophilization after identification and could not be tested. Thus, 78 isolates representing 28 species were each analyzed for 19 enzymes. A total of 113 complete tests were conducted owing to replication of tests, use of additional media for preparing the inocula of strains which did not grow well on Czapek solution agar, duplicate tests which were incubated as long as 24 h, and tests comparing enzymology of spore and mycelial inocula of selected strains. *Aspergillus amstelodami, Chaetomidium pilosum, Chaetomium elatum, Thielavia sepedonium, Xylohypha bantiana,* and the unidentified Ascomycete failed to grow well enough on Czapek solution agar to yield sufficient inocula for API ZYM tests and were therefore grown on potato dextrose agar and malt extract agar and then tested for enzymes. Other selected species were also grown on various media, and the enzymology was compared to inocula from Czapek solution agar. Results of tests on inocula of the same strain prepared on different media gave the same results except that the concentrations of one or two of the enzymes produced were in a few cases slightly higher when the growth medium was potato dextrose agar compared to malt extract agar. These differences could be related to improved growth of the molds and/or to the media composition. Bridge and Hawksworth (1984) also found some minor variations with different media. We also noted a few similar minor variations when the incubation time was extended for 24 h for strains of *R. nigricans.* Mycelial inocula produced the same enzymes as spore suspensions, although in smaller quantities.

Results of enzymatic activities based on identities of the isolates regardless of the pollen source are shown in Tables

Table III. Enzymes produced by Penicillia from pollen and bee bread.

Enzyme	*No. positive/number of isolates tested*					
	P. brevi-compactum P. chrysogenum		P. corylophilum	P. cyclopium P. crustosum		P. janthinellum
Alkaline phosphatase	3/5	1/1	14/14	4/8	9/11	1/1
Butyrate esterase	2/5	1/1	11/14	1/8	3/11	1/1
Caprylate esterase-lipase	5/5	1/1	14/14	6/8	11/11	1/1
Myristate lipase	1/5	0/1	0/14	1/8	0/11	0/1
Leucine aminopeptidase	5/5	1/1	14/14	8/8	11/11	1/1
Valine aminopeptidase	0/5	0/1	1/14	1/8	3/11	0/1
Cystine aminopeptidase	0/5	0/1	0/14	0/8	0/11	0/1
Trypsin	0/5	0/1	0/14	0/8	0/11	0/1
Chymotrypsin	0/5	0/1	1/14	0/8	0/11	0/1
Acid phosphatase	5/5	1/1	14/14	8/8	11/11	1/1
Phosphoamidase	5/5	1/1	14/14	8/8	11/11	1/1
α-Galactosidase	0/5	0/1	0/14	0/8	0/11	0/1
ß-Galactosidase	2/5	1/1	0/14	0/8	0/11	1/1
ß-Glucuronidase	0/5	0/1	0/14	0/8	0/11	0/1
α-Glucosidase	0/5	0/1	2/14	0/8	0/11	0/1
ß-Glucosidase	5/5	1/1	14/14	8/8	11/11	1/1
N-Acetyl-ß-glucosaminidase	5/5	0/1	13/14	8/8	11/11	0/1
α-Mannosidase	0/5	0/1	0/14	0/8	0/11	0/1
α-Fucosidase	0/5	0/1	3/14	0/8	0/11	0/1

III—VI. All 40 Penicillia tested produced leucine aminopeptidase, acid phosphatase, phosphoamidase, and ß-glucosidase; none produced cystine aminopeptidase, trypsin, α-galactosidase, ß-glucuronidase, or α-mannosidase (Table III). Most also produced alkaline phosphatase, caprylate esterase-lipase, and *N*-acetyl-ß-glucosaminidase. Enzymes produced by Penicillia in the highest concentrations (> 20 nmol) were acid phosphatase by *P. corylophilum*, *N*-acetyl-ß-glucosaminidase by *P. cyclopium*, and ß-glucosidase by *P. crustosum*.

All Aspergilli tested produced acid phosphatase, phosphoamidase, ß-glucosidase, and *N*-acetyl-ß-glucosaminidase; none produced myristate lipase, trypsin, chymotrypsin, ß-glucuronidase, α-mannosidase, or α-fucosidase (Table IV). Most also produced alkaline phosphatase, butyrate esterase, caprylate esterase—lipase, and leucine aminopeptidase. One of the *A. flavus* strains tested was var. *columnaris* and gave the same reactions as strains identified as *A. flavus*. Enzymes produced in the highest concentrations (> 20 nmol) by Aspergilli were *N*-acetyl-ß-glucosaminidase and ß-glucosidase by *A. niger*, alkaline phosphatase by *A. flavus*, and alkaline and acid phosphatases and ß-glucosidase by *A. versicolor*.

All Murocales tested produced leucine aminopeptidase, and most produced acid phosphatase and phosphoamidase (Table V). Otherwise these molds produced few enzymes. The only enzymes produced in high concentrations (≥ 20 nanomoles) were phosphoamidase by all strains of *R. nigricans* tested, acid phosphatase by 3 of the 4 *R. nigricans* strains, and leucine aminopeptidase by *Mucor racemosus*.

Table IV. Enzymes produced by Aspergilli from pollen and bee bread.

Enzyme	*No. positive/No. of isolates tested*				
	A. amstelodami	A. flavus	A. niger	A. ustus	A. versicolor
Alkaline phosphatase	0/1	3/3	4/4	1/1	1/1
Butyrate esterase	1/1	3/3	4/4	0/1	1/1
Caprylate esterase-lipase	1/1	3/3	4/4	0/1	1/1
Myristate lipase	0/1	0/3	0/4	0/1	0/1
Leucine aminopeptidase	1/1	3/3	4/4	0/1	0/1
Valine aminopeptidase	0/1	0/3	1/4	0/1	0/1
Cystine aminopeptidase	0/1	0/3	1/4	0/1	0/1
Trypsin	0/1	0/3	0/4	0/1	0/1
Chymotrypsin	0/1	0/3	0/4	0/1	0/1
Acid phosphatase	1/1	3/3	4/4	1/1	1/1
Phosphoamidase	1/1	3/3	4/4	1/1	1/1
α-Galactosidase	0/1	0/3	2/4	0/1	1/1
ß-Galactosidase	0/1	2/3	1/4	0/1	0/1
ß-Glucuronidase	0/1	0/3	0/4	0/1	0/1
α-Glucosidase	0/1	0/3	1/4	0/1	0/1
ß-Glucosidase	1/1	3/3	4/4	1/1	1/1
N-Acetyl-ß-glucosaminidase	1/1	3/3	4/4	1/1	1/1
α-Mannosidase	0/1	0/3	0/4	0/1	0/1
α-Fucosidase	0/1	0/3	0/4	0/1	0/1

Table V. Enzymes produced by Mucorales from pollen and bee bread.

Enzyme	*No. positive/No. of isolates tested*		
	Mucor sp.	Mucor racemosus	Rhizopus nigricans
Alkaline phosphatase	0/2	0/1	1/4
Butyrate esterase	0/2	0/1	0/4
Caprylate esterase-lipase	0/2	0/1	0/4
Myristate lipase	0/2	0/1	0/4
Leucine aminopeptidase	2/2	1/1	4/4
Valine aminopeptidase	0/2	0/1	1/4
Cystine aminopeptidase	0/2	0/1	1/4
Trypsin	0/2	0/1	0/4
Chymotrypsin	0/2	0/1	0/4
Acid phosphatase	1/2	1/1	4/4
Phosphoamidase	1/2	1/1	4/4
α-Galactosidase	0/2	0/1	0/4
ß-Galactosidase	0/2	0/1	0/4
ß-Glucuronidase	0/2	0/1	0/4
α-Glucosidase	0/2	0/1	0/4
ß-Glucosidase	0/2	0/1	1/4
N-Acetyl-ß-glucosaminidase	0/2	0/1	0/4
α-Mannosidase	0/2	0/1	0/4
α-Fucosidase	0/2	0/1	0/4

Of the other molds tested, most produced acid phosphatase and ß-glucosidase (Table VI). Alkaline phosphastase, butyrate esterase, caprylate esterase—lipase, leucine aminopeptidase, and phosphoamidase were produced by 52—67% of them. Enzymes produced in the highest concentrations (≥ 20 nmol) by various molds are as follows : alkaline phosphatase by *Aur. pullulans* from one-week bee bread; caprylate esterase—lipase by *C. herbarum* from corbicular pollen and by *C. cladosporioides;* leucine aminopeptidase by *Aur. pullulans* and *Peyronelia* sp.; acid phosphatase by *Alt. tenuis, Arthrinium phaeospermum, Aur. pullulans, C. cladosporioides, C. herbarum, Peyronelia* sp., *Scytalidium* sp., and *X. bantiana*; phosphoamidase by *X. bantiana*; α-galactosidase by *Aur. pullulans* from one-week bee bread and by *C. cladosporioides* from corbicular pollen; ß-glucosidase by *Alt. tenuis* and *T. sepedonium* and by *C. cladosporioides* from 6-week bee bread; and α-fucosidase by *C. cladosporioides* from floral and corbicular pollen.

Enzymology of the 78 molds tested is presented in Table VII on the basis of the pollen sources of the isolates. No molds produced trypsin, ß-glucuronidase, or α-mannosidase. Few produced myristate lipase, and only one produced chymotrypsin; these isolates were from corbicular pollen. Valine aminopeptidase and cystine aminopeptidase were not produced by isolates from 3-week bee bread, nor was the latter enzyme produced by molds from corbicular or 6-week bee bread. Few molds from other pollen sources produced these two peptidases. Of the glycosidases, α-glucosidase and α-fucosidase were not associated with molds from either 3-week or 6-week bee bread and

were produced by few isolates from other pollen sources. Both α- and ß-galactosidases were produced by a higher percent of molds tested from floral pollen than from other pollen sources. No molds from corbicular pollen produced ß-galactosidase.

However, most molds from all pollen sources produced caprylate esterase—lipase, leucine aminopeptidase, acid phosphatase, phosphoamidase, ß-glucosidase, and *N*-acetyl-ß-glucosamidase. A high percent of the isolates (≥ 50%) from all sources gave positive reactions for alkaline phosphatase. This was also the case with butyrate esterase except for those molds from one-week bee bread. Therefore, pollen molds produced enzymes involved in protein, lipid, and carbohydrate metabolism.

Discussion

Seventy percent of the molds identified from almond pollen and bee bread were Aspergilli, Mucorales, and Penicillia. We have previously isolated from apiarian sources in Arizona most of the species of Aspergilli and Penicillia found in pollen and bee bread as well as *Aur. pullulans, C. claedosporioides,* Mucorales, and *Peyronelia* sp. (Gilliam and Prest, 1972, 1977, 1987; Gilliam *et al.,* 1974, 1977, 1988). Frequent isolates in the present study which are new records of molds from apiarian sources in Arizona are *Alt. tenuis, Penicillium crustosum,* and *R. nigricans.*

Molds which were not present in floral pollen but were frequent isolates from corbicular pollen and bee bread may have been introduced by the bees during collection and storage. The most obvious examples are *Aur. pullulans, P. corylophilum, P. crustosum,* and *R. nigricans.* Conversely, *Mucor* sp., the dominant mold in floral pollen, was eliminated in corbicular pollen and bee bread. Thus, as with yeasts (Gilliam, 1979a) and *Bacillus* ssp. (Gilliam, 1979b), the mold flora of corbicular pollen and bee bread may be the result of microbial inoculations by bees and chemical changes in pollen resulting from additions by bees from regurgitation of honey sac contents and secretions of glands as well as microbial fermentation which allow some species but not others to survive. Even though molds were more numerous than yeasts or *Bacillus* ssp. in our samples, pollen is rarely overgrown by molds. Potential microbial spoilage of pollen provisions may be controlled by antibiotic substances produced by the normal microflora, bees, pollen, and/or honey.

Klungness and Peng (1983) examined corbicular pollen and bee bread with scanning electron microscopy and found no visible evidence of digestion or damage to pollen grain walls. They concluded that microorganisms associated with bee bread do not cause destruction of pollen intine or the cytoplasm, that substances bees add to pollen during collection and storage function as a preservative, and that the regurgitation added to pollen probably allows growth of some microorganisms and inhibits the growth of others. They observed that the few fungal spores that germinated produced hyphae less than 10 µm in length.

If microorganisms are responsible for fermentation and the accompanying chemical changes of pollen stored in comb cells by honey bees, the molds may be a component of the required microbial complement. They could contribute antibiotics, organic acids and enzymes, products for which they are utilized industrially. These compounds may limit the growth of deleterious microorganisms and provide enzymes for utilization of nutrients.

Table VI. Enzymes produced by other molds from pollen and bee bread.

Enzyme	*No. positive/No. of isolates tested*						
	Alternaria tenuis	Arthrinium phaeospermum	Aureobasidium pullulans	Chaetomidium pilosum	Chaetomium elatum	Cladosporium cladosporioides	Cladosporium herbarum
Alkaline phosphatase	1/2	1/1	2/2	1/1	1/1	0/5	1/2
Butyrate esterase	2/2	0/1	2/2	0/1	0/1	4/5	1/2
Caprylate esterase-lipase	2/2	1/1	2/2	0/1	0/1	5/5	1/2
Myristate lipase	0/2	0/1	0/2	0/1	0/1	1/5	0/2
Leucine aminopeptidase	2/2	0/1	2/2	0/1	0/1	5/5	1/2
Valine aminopeptidase	0/2	0/1	0/2	0/1	0/1	0/5	0/2
Cystine aminopeptidase	0/2	0/1	0/2	0/1	0/1	0/5	0/2
Trypsin	0/2	0/1	0/2	0/1	0/1	0/5	0/2
Chymotrypsin	0/2	0/1	0/2	0/1	0/1	0/5	0/2
Acid phosphatase	2/2	1/1	2/2	0/1	0/1	5/5	1/2
Phosphoamidase	2/2	1/1	2/2	0/1	0/1	4/5	1/2
a-Galactosidase	2/2	0/1	2/2	0/1	0/1	5/5	1/2
ß-Galactosidase	2/2	0/1	0/2	0/1	0/1	0/5	0/2
ß-Glucuronidase	0/2	0/1	0/2	0/1	0/1	0/5	0/2
α-Glucosidase	2/2	0/1	0/2	0/1	0/1	0/5	0/2
ß-Glucosidase	2/2	1/1	2/2	1/1	1/1	5/5	1/2
N-Acetyl-ß-glucosaminidase	0/2	1/1	0/2	0/1	1/1	4/5	0/2
α-Mannosidase	0/2	0/1	0/2	0/1	0/1	0/5	0/2
α-Fucosidase	0/2	0/1	0/2	0/1	0/1	2/5	0/2

Table VI. (continued)

Enzyme	*No. positive/No. of isolates tested*						
	Paecilomyces varioti	Peyronelia *sp.*	Scopulariopsis brevicaulis	Scytalidium *sp.*	Thielavia sepedonium	Xylohypha bantiana	*Unidentified Ascomycete*
Alkaline phosphatase	1/1	1/1	1/1	1/1	0/1	0/1	1/1
Butyrate esterase	1/1	0/1	0/1	1/1	0/1	0/1	0/1
Caprylate esterase-lipase	1/1	1/1	0/1	1/1	0/1	0/1	0/1
Myristate lipase	0/1	0/1	0/1	0/1	0/1	0/1	0/1
Leucine aminopeptidase	1/1	1/1	0/1	1/1	0/1	0/1	0/1
Valine aminopeptidase	0/1	1/1	0/1	0/1	0/1	0/1	0/1
Cystine aminopeptidase	0/1	0/1	0/1	0/1	0/1	0/1	0/1
Trypsin	0/1	0/1	0/1	0/1	0/1	0/1	0/1
Chymotrypsin	0/1	0/1	0/1	0/1	0/1	0/1	0/1
Acid phosphatase	1/1	1/1	1/1	1/1	1/1	1/1	0/1
Phosphoamidase	1/1	1/1	0/1	1/1	0/1	1/1	0/1
α-Galactosidase	0/1	0/1	0/1	0/1	0/1	0/1	0/1
ß-Galactosidase	0/1	0/1	1/1	0/1	0/1	0/1	0/1
ß-Glucuronidase	0/1	0/1	0/1	0/1	0/1	0/1	0/1
α-Glucosidase	0/1	1/1	0/1	0/1	0/1	0/1	0/1
ß-Glucosidase	0/1	1/1	1/1	0/1	1/1	1/1	1/1
N-Acetyl-ß-glucosaminidase	0/1	1/1	0/1	0/1	1/1	1/1	0/1
α-Mannosidase	0/1	0/1	0/1	0/1	0/1	0/1	0/1
α-Fucosidase	0/1	0/1	0/1	0/1	0/1	0/1	0/1

Indeed, we have found Aspergilli, Mucorales, Penicillia, and other molds in bee bread and guts of worker bees which inhibit the growth of the chalkbrood fungus (Gilliam *et al.*, 1988).

Enzymology of our isolates revealed that the major phosphatase was acid phosphatase, although alkaline phosphatase was produced by most isolates except the Mucorales. Molds apparently are not able to participate in the initial breakdown of long-chain fatty acids as evidenced by the lack of myristate lipase. However, they did produce butyrate esterase and caprylate esterase—lipase which act on shorter chain fatty acids. Leucine aminopeptidase was the major aminopeptidase. Molds did not produce trypsin or chymotrypsin. Phosphoamidase was produced by most isolates tested.

Results for glycosidases revealed that Mucorales were quite unreactive. However, most other molds produced ß-glucosidase which hydrolyzes carbohydrates such as cellobiose, salicin, amygdalin, and gentibiose. *N*-Acetyl-ß-glucosaminidase was also produced by most molds. This enzyme is involved in hydrolysis of chitin. ß-Galactosidase which hydrolyzes lactose and α-glucosidase which hydrolyzes sucrose, maltose, trehalose, and melizitose were not produced by most of the molds.

In summary, molds as normal microflora in pollen and bee bread have received little attention. However, our results indicate that because they represented 38% of the total number of microorganisms we isolated (Gilliam, unpublished data), produced a variety of enzymes, and are well known for production of secondary metabolites such as antibiotics, phenolic compounds, terpenes, steroids, and polysaccharides as well as enzymes, they

Table VII. Percent of pollen and bee bread molds tested that produced various enzymes.

Enzyme	*Source of molds*				
	Floral pollen	*Corbicular pollen*	*1-Week bee bread*	*3-Week bee bread*	*6-Week bee bread*
Alkaline phosphatase	50	70	82	64	75
Butyrate esterase	50	52	36	64	67
Caprylate esterase-lipase	80	87	86	64	67
Myristate lipase	0	13	0	0	0
Leucine aminopeptidase	100	96	86	82	75
Valine aminopeptidase	20	13	5	0	17
Cystine aminopeptidase	10	0	5	0	0
Trypsin	0	0	0	0	0
Chymotrypsin	0	4	0	0	0
Acid phosphatase	90	100	95	100	75
Phosphoamidase	90	100	86	100	75
α-Galactosidase	30	17	14	9	17
ß-Galactosidase	40	0	9	9	17
ß-Glucuronidase	0	0	0	0	0
α-Glucosidase	20	4	9	0	0
ß-Glucosidase	80	91	91	82	92
N-Acetyl-ß-glucosaminidase	70	78	68	73	67
α-Mannosidase	0	0	0	0	0
α-Fucosidase	10	9	9	0	0

merit more intensive study. Even if mold spores that germinate in corbicular pollen and bee bread produce short hyphae (Klungness and Peng, 1983), our results with selected isolates of various genera confirmed those with Penicillia by Bridge and Hawksworth (1984) that mycelia inocula produce the same enzymes, although in reduced amounts, as spore suspensions of the same strain. With this publication and those on yeasts (Gilliam, 1979a) and *Bacillus* ssp. (Gilliam, 1979b), we have now reported 77% of the total isolates from our almond pollen and bee bread samples. The remaining microorganisms will be described in a future publication.

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