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CHARACTERIZATION OF THE YEASTS (*BLASTOMYCETES*) IN SOME FIJIAN HONEYS

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A suitable culture medium with a composition of 50% honey and 2% agar was developed. This gave optimum honey yeast and bacterial population growth. An identification of the major *Blastomycetic* (yeast) populations for seven Fijian honeys appear to have representatives of all the major yeast aspecies observed in foreign honeys, more than normal *Brevibacterium* and *Gluconobacter* bacterial population were detected.

INTRODUCTION

One of the most serious concerns of the apiculturist is honey fermentation [33]. This problem, when it occurs, has important economic consequences particularly regarding sales. Therefore, a knowledge of the organisms which induce this fermentatio is desirable [31]. Such organisms are generally osmophilic yeasts, the *Zygosaccharomyces*. These thrive in very concentrated suger environments such as honey [5]. Less well documented are the *Brevibacterium* bacteria, whose population is more numerous (150-500/g) in freshly extracted honeys than in matured honeys. Thus, bacteria counts drop to approximately 20-40/g after 48 hours, and in some honeys to as low as zero after one week [27].

In this paper we describe the major species of *Blastomycetes* (yeasts) and bacteria in Fijian honeys. Before this, the best conditions for the growth of these microorganisms had to be established and this is also reported.

EXPERIMENTAL

All honey samples investigated were kept in cold storage (4 ± 1°C) until required. Sterlization and handling of the honeys was according to the methods described by [5]. Honey substrates were 25, 50, 75 and 100% honey (diluted with sterile water), containing 2% agar (Oxoid, agar No. 1, Code L11). A sample from 4 miles Nasinu was used for this. A culture stock of 1.44 litres, foreach gradient, was made and a total of 72 plates prepared. Sterlin gamma-irradiated petri dishes were used and samples introduced by the "looping-out" method [5]. All plating was carried out in aseptic conditions [5] and plates prior to "looping" were kept in a Bassaire ultraviolet sterilizer box (l.c. 6, 17). The culture stock solutions were sterilized in a Griffin steam sterilizer (model No. 1646), at a temperature of 120°C and guage pressure of 1.02 atmospheres for 15 minutes. Incubation, employing a Gallenkamp 1H-285, Model No. 4B/6080/B incubator, was at 24.8°C. An Astell petri dish viewer was used for culture identifications and all yeasts were identified and confirmed by the methods outlined by Lodder et al. [16]. Polymorphic bacteria were confirmed by the methods of Rodriguez-Navarro and Ruiz-Argueso [27, 28].

RESULTS AND DISCUSSION

CULTURE MEDIA INVESTIGATIONS

The culturing and isolation of individual strains of non-osmophilic yeasts and bacteria have been well established [30]. However, the establishment of a successful madium for the growth of strains which are sugar-tolerant (osmophilic) is more difficult [6].

The first recommended medium for the isolation of osmophilic yeasts was by Kroemer and Krumbholz (l.c. 6). This medium had a 50% sugar concentration. The development of a medium containing only glucose as the carbon source has been advocated by Nickerson and Mankowski (l.c. 6), particularly for yeasts of the genus Candida. Furuta and Okimoto was by Kroemer and Krumbholz (l.c. 6). This medium had a 50% sugar tolerant organisms grew on this medium. They recommended that additional sugars be added to glucose. Lochhesd and Heron [15], investigating the yeast populations of Canadian honeys found that osmophilic yeasts from fermenting honey grew best in an agar medium containing 60-67% honey.

In our investigation, a medium for the optimum growth of osmophilic yeasts and bacteria was developed, with honey as the sole carbon source. Cook [6] has commented that in the isolation of "organisms from foods

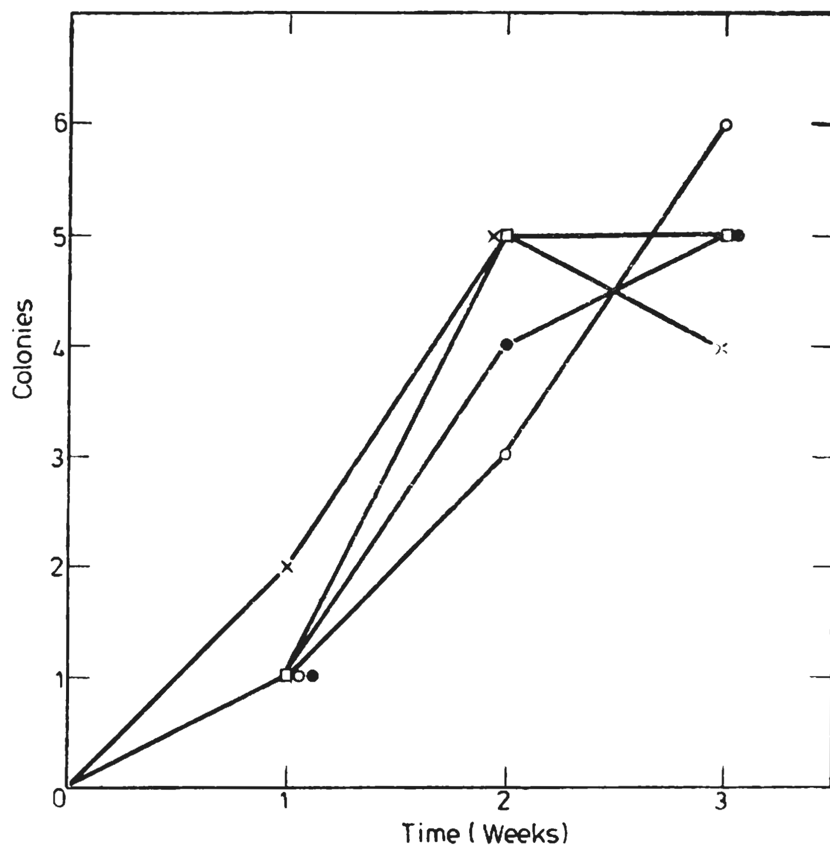

Fig. 1. Growth curves of *Blastomycetes* (yeasts) on honey substrates containing 2% agar: (□) 25%; (○), 50%; (×), 75%; (·), 100% honey

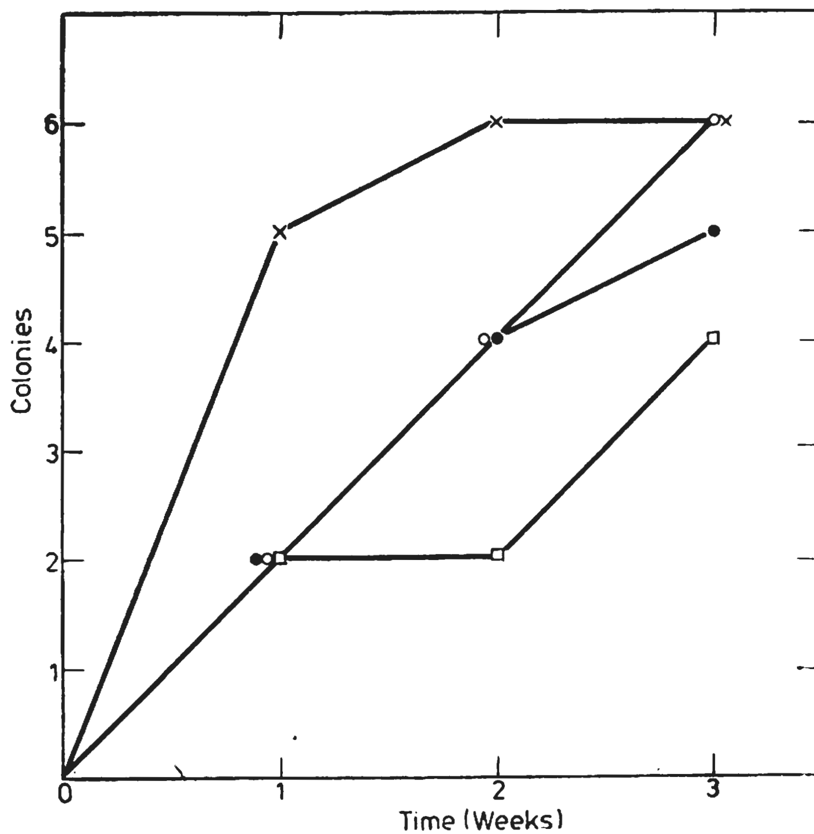

Fig. 2. Growth curves of bacteria on honey substrates containing 2% agar: (□), 25%; (○), 50% (×), 75%; (·), 100% honey

having high concentrations of salt or sugar, it is often sufficient to isolate them on a medium of similar composition".

Four honey substrate compositions of 25, 50, 75 and 100% respectively were tested. A sample from 4 miles Nasinu was diluted to these compositions with sterile water and 2% agar added. These cultures after sterilization were inocculated with the Ovalau honey and incubated for a period of three weeks, with weekly observational periods to ascertain growth.

The optimum incubation temperature for osmophilic yeasts has been reported by Cook (1958) to be 20-25°C. The maximum temperature which can be used may vary between 30° and 46-47°C. In this study the incubation temperature was 24.8°C.

The average number of colonies, for both yeasts and bacteria, were assessed and the groeth curves (Figures 1 and 2) for each medium determined.

The growth curves, for both the osmophilic yeasts and bacteria, have the characteristic lag, rapid growth and death phases [30]. The 25% medium, for both yeasts and bacteria, behaves poorly. Yeasts, in this medium shown no exponential growth until after two weeks incubation, and the bacteria, over a similar time, exhaust this substrate and remain constant (static growth). The 75% medium sustains rapid growth, upto two weeks incubation, for both yeasts and bacteria. The former attain a state of constancy senile or terminal phase) whereas the latter rapidly decrease in number (death phase). The 100% medium responds similarly, for both yeasts and bacteria, and after three weeks incubation a state of constancy is attained. The 50% medium, on the other hand, displays ideal growing conditions, for both yeasts and bacteria, for exponential growth is maintained even after three weeks incubation.

Consequently, the medium with a composition of 50% honey was chosen as the best substrate on which to grow yeast and bacteria. New cultures with this composition were prepared and each honey culture was grown for an incubation period of three weeks, before the colonies were identified.

YEAST AND BACTERIA IDENTIFICATION

The number of species of osmophilic yeasts in honeys are variable. Aoyagi and Oryu [3] investigating 16 Japanese honeys found only 6 species of which one, Nematospora ashbya gossypii, was doubtful. On the other hand, Kumbhojkar [10] found 193 strains of osmophilic and osmotolerant yeasts in Indian honeys; 24 of which were assigned to the genus *Saccharomyces*. Malan and Marletto [18] also found large numbers of yeast strains (199) in Italian honeys. The species *Torulopsis mogii* and *Saccharomyces rouxii* were predominant in all samples.

Table 1. Yeasts and bacteria identified in Fijian honeys

Sample	pH[a,b]	Moisture (%)[b]	Microbial composition[c]	Reference[d]
Nasinu	3.80	18.4	*Saccharomyces torulosus*	A
			Zygosaccharomyces sp[e]	N
			Brevibacterium	R(a)
Nausori	3.77	18.5	*Zygosaccharomyces mellis*[e]	F(a)
			Brevibacterium	R(a)
Lautoka (A)	f	f	*Torula mellis*	F(a)
			Saccharomyces globosus	F(b)
			Brevibacterium[e]	R(a)
			Gluconobacter	R(b)
Lautoka (B)	4.01	16.8	*Torulopsis mogii*	M
			Schizosaccharomyces octosporus	L(a)
			Zygosaccharomyces barkeri	L(b)
			Brevibacterium	R(a)
			Gluconobacter[e]	R(b)
Lautoka (C)	f	17.1	*Zygosaccharomyces rechteri*	L(b)
			Saccharomyces rouxii	F(a)
			Brevibacterium[e]	R(a)
			Gluconobacter[e]	R(b)
Lautoka (D)	f	16.3	*Candida reukaufii*[e]	S
			Torulopsis mogii	M
			Brevibacterium	R(a)
			Gluconobacter	R(b)
Ovalau	3.96	18.0	*Torula mellis*	F(a)
			Saccharomyces globosus	F(b)
			Zygosaccharomyces mellis	F(a)
			Brevibacterium[e]	R(a)

a. 5% solution
b. Pocini and Wimmer (1983)
c. Identified by the taxonomic methods of Lodder (1971).
Grown on a 50% honey: sterile water medium with 2% agar and incubated for 3 weeks at 24.8°C.
d. References used to establish the true generic names: A, Aoyagi and Oryu (1968); F(a), Fabian and Quinet (1928); F(b), Furuta and Okimoto (1975); L(a), Lochhead and farrell (1931); L(b), Lochhead and Heron (1929); M, Malan and Marletto (1973-74); N, Nussbaumer (1910); R(a), Rodriguez-Navarro and Ruiz-Argueso (1970); R(b), Ruiz-Argueso and Rodriguez-Navarro (1973); S, Spencer et al. (1970).
e. Trace only.
f. Insufficient sample.

In this study, 11 species of osmophilic yeasts were found as well as two bacterial genera *Gluconobacter* and *Brevibacterium* (Table 1). The latter being the most abundant honey bacterial genus detected; was found in all samples investigated whereas the *Gluconobacter* genus was found in only those honeys from Lautoka.

Lochhead and Farrell [14] established that only species of *Saccharomyces* and *Zygosaccharomyces* were capable of causing fermentation in honey, whereas *Schizosaccharomyces* and *Torulopsis cause* no fermentation. Of the often the seven honeys investigated, four contained species

of *Saccharomyces* and five honeys contained *Zygosaccharomyces species* (Table 1).

The only *Schizosaccharomyces species* (*S. octosporus*) was found in the Lautoka (B) sample, whereas two honeys (Lautoka (B) and (D)) contained a Torulopsis species (*T. mogii*).

Other yeast species were *Torula*, present in two honeys (Lautoka (A) and Ovalau) as *T. mellis* and a *Candida* species (*C. reukaufii*) in Lautoka (D). The genus *Torula* has a low fermenting ability (Tanner, 1938), whereas the Candida species has been found to demonstrate either fermentation or no fermentation [6].

These latter two yeast species have been isolated from flowers. Capriotti (l.c. 6) found numerous species of *Candida* and *Torulopsis* in Italian flowers. Schollhorn (l.c. 6) isolated 10 different *Torulae* species as well as *C. reukaufii* from flowers in Geneva. Hautmann (l.c. 6) and Gruss (l.c. 6) both have established that *C. reukaufii* grows well in nectars of various flowers.

The verification that certain yeasts in fermented honey were identical with yeasts isolated from flowers has been discussed by Cook [6]. Osmophilic yeasts are nearly ubiquitous on the bodies of bees, in nectar, soil in apiaries and in extracting and storage areas [33]. The species *C. reukaufii* has been found in the stomach of bees and bumblebees as well as in flowers and fruits [6].

It is now well established [2, 31] that the bee is the immediate source of honey yeasts. The bacteria in honey have also been soleley attributed to the bee as their vehicle of introduction [27].

Once the yeasts or bacteria are introduced into the honey, their survival and multiplication has been shown by many workers (32, 1, Schachinger and Heiss, l.c. 6, English, l.c. 6, Schelhorn, l.c. 6, Scarr, l.c. 6 and Vas l.c. 6) to be dependent upon the moisture content of the honey, and not on the nature and amount of carbohydrates present.

The amount of water in honey is important for its stability against fermentation and granulation [33]. The minimum water content of honey, necessary to support a fermentative yeast population is uncertain. Many values have been reported, for example: 17.5-18.0% [33]; 21.5% [21], and 22.5-26.0% [19]. Lochhead [13] has tabulated a range of moistures based on yeast counts for 319 honeys (Table 2).

The moisture contents for the Fijian honeys were in the range 16.3-18.5% (Table 1). The honeys with a moisture content greater than 18.0% had large numbers of *Saccharomyces* and *Zygosaccharomyces* species; yeasts causing fermentation (vide supra). Honey containing less than 18.0% moisture contained relatively few fermenting yeast and large colonies of *Schizosaccharomyces* and *Torulopsis* species (non-fermenting yeasts). Brevibacterium and Gluconobacter bacteria both favoured higher moistures (i.e. $>$ 18.0%).

Table 2. Moisture content — indicator of honey fermentation*

Moisture content (%)	Fermentation
Below 17.1	None
17.1-18.0	None, if yeast count 1000/gm.
18.1-19.0	None, if yeast count —10/gm.
19.1-20.0	None, if yeast count — 1/gm.
Above 20.0	Always

* Data of Lochhead (1933) based on 319 honeys

Honey is hygoscopic, but due to its voscosity the absorption of water is confined to the surface layer. Consequently, osmophilic yeasts and bacteria can grow on the surface when, in the absence of this factor, no growth should occur (Sacchetti, l.c. 6; 7; 19).

Honey acidity is also a contributing factor in osmophilic yeast development [33]. White et al. [34] reported that U.S. honeys had an average pH of 3.91, with variations from 3.42 to 6.10. The pH values for the Fijian honeys (Table 1) average about 3.84. This honey acidity enhances stability against microbial attact provided moisture levels are favourable. The Fijian honeys, in terms of their overall yeast populations, moisture content and pH are relatively stable to fermentation. However, becouse of the large populations of osmophilic bacteria (*Gluconobacter* and *Brevibacterium* species) three honeys (Nasinu, Nausori and Ovalau) became extremely turbid, when solutions were left at room temperature for 24 hours.

It is conceivable, that this higher than normal level of osmophilic bacteria may be due to unclean handing and collecting of the samples. Gassparin and Vorwohl [9] have demonstrated for Iranian honeys that hygiene when harvesting honey, is most important for prolonging storage life, minimizing fermentation and preventing turbidity.

As extracted, "raw" honey contains extraneous matter such as pollen, bits of wax and more importantly variable amounts of sugartolerant yeasts [33], which once established induce honey spoilage thus reducing marketabily [6]. The methods generally used to reduce microbial spoilage are either heat or cold treatments. The former treatment has been widely studied (22, and references therein). Fabian and Quinet [7] have shown that heating honey at 60°C for 5-10 minutes caused the destruction of *Saccharomyces* and *Zygosaccharomyces* species. The heating process must be carefully controlled to prevent secondary deterioration of the sugars [9] Langridge [11] has determined that for a normal honey processing plant a safe temperature of 45°C can be employed without causing serious sugar deterioration. Liebl [12] has reduced fermentation by ultrasonication, at 18.000 Hz with a temperature of 10-38°C. He has shown this method to be superior to heating and less time consuming.

Cold (i.e. below 10°C) treatment has been recommended for unprocessed honey to prevent fermentation [30]. However, because of the need for expensive refrigeration equipment, this method is not widely used commercialy.

The best method, for the elimination of fermentation, is to protect the honey from atmospheric moisture. The cheapest and most widely used technique is storage in sealed sterilized containers [23]. The problems associated with this area of honey starage have been reviewed by Patwardhan and White [25] and by Bland [4].

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CHARAKTERYSTYKA DROŻDŻY (*BLASTOMYCETES*) WYIZOLOWANYCH Z MIODÓW WYSPY FIDŻI

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S t r e s z c z e n i e

Przebadano siedem próbek miodu miejscowej produkcji oraz wpływ zawartości miedzi (25%, 50%, 75% i 100%) w pożywce z 2% agarem na wzrost drożdży i bakterii. Najlepsze efekty osiągano stosując pożywki z 50% udziałem miodu (rys. 1 i 2). Zidentyfikowano 10 gatunków drożdży oraz 2 rodzaje bakterii *Brevibacterium* i *Gluconobacter*. Spośród drożdży zidentyfikowano: *Saccharomyces torulosus, Zygosaccharomyces mellis, Saccharomyces globosus, Torulopsis moggi, Schizosaccharomyces octosporus, Zygosaccharomyces barkeri, Zygosaccharomyces rechteri, Saccharomyces rouxii, Candida reukaufii, Torula mellis, Torulopsis mogii* (tab. 1).