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Role of Pectinolytic Yeasts in the Degradation of Mucilage Layer of *Coffea robusta* Cherries

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ABSTRACT

AGATE, A. D. (Indian Institute of Science, Bangalore, India), AND J. V. BHAT. Role of pectinolytic yeasts in the degradation of mucilage layer of *Coffea robusta* cherries. Appl. Microbiol. **14**:256–260. 1966.—Pectinolytic yeasts, *Saccharomyces marxianus*, *S. bayanus*, *S. cerevisiae* var. *ellipsoideus*, and *Schizosaccharomyces* sp., predominated in the natural fermentation of coffee cherries of *Coffea robusta* variety grown in Chikmagalur district of Mysore State, India. These yeast species were found on the cherry surfaces, and evidence was adduced to show that the natural fermentation of coffee was the result of activity of microflora from the cherry surface itself rather than that of flora of air or water. Incorporation of pure cultures of *Saccharomyces* species was shown to aid the process when a mixture of all three species was used. An enzyme preparation from the *Saccharomyces* species was observed to hasten the mucilage-layer degradation.

An important and noticeable change occurring in the fermentation of coffee is the degradation of the mucilage layer that surrounds the beans. In fact, the fermentation is adjudged "finished" when the parchment on touch "feels hard" and is free from slippery mucilage. The mucilage layer contains a large amount of pectic substances which are degraded during the fermentation. Whether or not this degradation was brought about by the enzymes from cherries themselves or by the microbial load on the exterior surfaces was not clear at the time this investigation was undertaken 2 years ago. From the reports of Frank and Dela Cruz (2) and Frank, Lum, and Dela Cruz (3), it appeared that microorganisms on the cherry surfaces have a part to play and that the decomposition of the mucilage layer is attributable to certain gram-negative, lactose-fermenting bacteria present on the cherry surface as well as in the plantation soil of Kona district of Hawaii. They identified the demucilaging strains as *Erwinia dissolvens*, and demonstrated the ability of the supernatant fluid of liquid cultures to decompose the mucilage layer of coffee cherries. This observation is in agreement with that of Vaughn et al. (14), who first reported on the pectinolytic activity of coliform bacteria associated with Brazilian coffee cherries.

The method in practice for demucilaging coffee beans in Chikmagalur district of Mysore (India) is the wet method of fermentation. With this procedure, the deskinned coffee cherries are allowed to undergo a natural fermentation for about 70 hr, during which period the mucilage layer is degraded adequately to be easily detached from the beans in the running water. The beans then are ready for drying operations (1).

The purpose of the present communication is to show that certain yeasts associated with the coffee cherries not only possess high pectinolytic activity but do seem to have a vital role in the demucilaging of coffee as practiced in this country. Mention is made here of the reported presence of yeasts in the process (7, 9), though their role has so far remained obscure.

MATERIALS AND METHODS

Coffee cherries and mucilage. Three samples of cherries of *C. robusta* (75 to 100 cherries weighing 500 to 600 g in each sample) and eight mucilage samples (each approximately 50 ml) at different stages of fermentation were collected directly into sterile tubes from the Central Coffee Research Station. Balehonnur, Chikamagalur District of Mysore State, India. The cherries were picked from the several shrubs scattered in three different areas of the farm. The samples were examined within 24 hr of collection, and when this was not possible the samples were stored in the cold prior to testing.

Microflora of cherry surface and mucilage. Ripe, unblemished cherries, eight in each tube and in du-

plicate sets, were shaken in sterile tap water, suitably diluted, and plated on nutrient agar, *p*H 7.0 (NA) and glucose yeast-extract-agar, *p*H 5.0 (GYE). After 24 to 48 hr of incubation at 30 C, enumeration and isolation of representative colonies of yeasts was achieved from these plates. All the mucilage samples in 5-ml portions were examined in the same manner.

Enrichments were set up with portions of mucilage samples as inocula in a medium containing Seitz-filtered pectin or polygalacturonic acid (both from Eastman Organic Chemicals, Rochester, N.Y.) at the 1% level as the only carbon source in a mineral-based medium.

Fermentation of coffee cherries. The skin was removed from the coffee cherries under aseptic conditions, and the beans were placed in sterile flasks. Perfect skinning could not be achieved, as tiny bits of skins adhered to the beans. The skinned cherries were covered with sterile water and allowed to undergo fermentation at room temperature (20 to 26 C). At regular intervals, samples were removed for *p*H measurements, microbial counts, and evaluation of mucilage-layer degradation. The extent of mucilage degraded was evaluated quantitatively by estimating the percentage loss in the total pectic substances obtained as calcium pectate (5).

Microflora of air or tap water in the fermentation of coffee cherries. The cherries were surface-sterilized by immersion in a 1:500 solution of mercuric chloride for 10 min, followed by thorough rinsing in sterile water to remove traces of mercuric chloride (Khambata, Ph.D. Thesis, Univ. of Bombay, Bombay, India, 1954). One set of aseptically skinned cherries was allowed to ferment in sterile tap water under aseptic conditions, and the other was exposed to air for 10 min prior to incubation. A third set was prepared for fermentation with tap water instead of sterile water. The extent of mucilage-layer degradation and microbial population of each set was determined by the methods described above.

Identification of the isolates. The yeast isolates were identified by reference to the work of Wickerham (15) and Lodder and Kreger Van-Rij (6). Their morphological and growth characteristics were followed for 4 days at 30 C and for 30 days at room temperature in malt extract broth and on malt extract-agar. Slide cultures were also prepared for the purpose. The sporulation was tested in the various recommended media. For sugar assimilation and fermentation studies, glucose, galactose, sucrose, maltose, lactose, and raffinose were used. Assimilation of potassium nitrate, utilization of ethyl alcohol as a sole carbon source, and splitting of arbutin were the other tests made. In addition, formation of starch by germinating spores, ability to grow at 40 C, and fermentation of inulin were also studied.

The bacterial isolates were studied by procedures described in the *Manual of Microbiological Methods* (13). *Bergey's Manual of Determinative Bacteriology* was used for their identification.

Pectinolytic activity of the isolates. The organisms were grown in media containing pectin or polygalacturonic acid (0.5%) for 4 days at 30 C, and the culture fluids thereafter were tested for pectinolytic activity after 6 hr of incubation.

The polygalacturonase (PG) activity was determined by a modification of the Willstätter-Schudel method (5) and that of pectin methylesterase (PE) by a method similar to Smith's modification (12). Pectin/polygalacturonic acid *trans*-eliminase (PTE/PATE) was detected by the method of Nagel and Vaughn (8). The percentage decomposition of pectin was estimated by Kaiser's (D.Sc. Thesis, Univ. of Paris, Paris, France, 1961) method.

Ability of pure cultures of yeasts to degrade mucilage. The dominant, strongly pectinolytic *Saccharomyces* cultures were inoculated individually or in various combinations into tubes of surface-sterilized skinned cherries kept in sterile tap water under aseptic conditions. The external appearance of the beans, along with the disappearance of stickiness from seeds, and the flavor of the ferment were considered as markers of an efficient fermentation. Chemically, estimation of pectic substances as calcium pectate (5) before and after the fermentation was considered a useful guide in following the process.

Yeast enzyme preparation in the process. S. marxianus, S. bayanus, and *S. cerevisiae* var. *ellipsoideus,* isolated from the cherries, were grown in a medium containing polygalacturonic acid (0.5%) for 4 days at room temperature. The growth was hastened by keeping the cultures on a rotary shaker (250 rev/min; 5-cm eccentric throw). Acetone powder extracts were obtained from these culture filtrates (10). The preparations from each species were dialyzed against distilled water for 24 hr at 4 C, and were then pooled together. The pooled yeast enzyme preparation (0.2%) was incorporated into skinned cherries undergoing natural fermentation. For comparison, commercial mold enzyme preparations, Benefax and Pectinase (Nutritional Biochemicals Corp., Cleveland, Ohio), were used at the 0.2% level on two other sets of cherries. The same criteria used previously were employed for recording the progress of fermentation, except that in the present experiment the rate of decomposition was measured at every 2-hr period.

RESULTS AND DISCUSSION

Microflora of mucilage and cherry surface. At every stage, there were more yeasts than bacteria in mucilage samples. On NA and GYE plates, the proportion of yeast to bacterial colonies at the end of 70 hr was of the order of 10:1 (Table 1). The yeast isolates were identified as *S. marxianus, S. bayanus, S. cerevisiae* var. *ellipsoideus,* and *Schizosaccharomyces* sp., in that order of dominance. The bacteria encountered belonged to the genera *Streptococcus, Pseudomonas, Flavobacterium,* and *Proteus.*

The yeasts were dominant on the cherry surfaces. The proportion of yeasts varied from 47 to 52% of the total surface population, and the species were identical to those obtained from mucilage.

TABLE 1. *Microbial population of mucilage and coffee cherries**

Samples	No. of organisms			
	Nutrient agar		Glucose yeast-extract-agar	
	Yeasts	Bacteria	Yeasts	Bacteria
Mucilage				
Initial, *p*H 6.8	64	66	125	156
4 hr, *p*H 6.4	180	80	292	281
12 hr, *p*H 6.2	586	156	648	392
24 hr, *p*H 6.0	823	351	923	350
36 hr, *p*H 5.9	1,260	436	1,520	300
48 hr, *p*H 5.8	2,650	510	2,360	278
60 hr, *p*H 5.5	4,850	528	2,950	264
70 hr, *p*H 5.1	6,010	569	3,000	255
Cherry				
Sample 1	255	250	260	236
Sample 2	230	247	253	240
Sample 3	237	238	240	232

* Results with mucilage samples are expressed as organisms per milliliter of liquid $\times 10^3$; with cherry samples, as organisms per cherry $\times 10^3$.

Fermentation of coffee cherries. The fluctutation in microbial population presented a typical pattern (Table 2). During the later stages, the bacterial counts remained more or less constant, whereas the yeast counts increased steadily. The population densities recorded in the laboratory tallied with those of mucilage samples brought for analysis from the Coffee Research Station, Balehonnur. The yeasts encountered in the liquor were also identical to those occurring in the processing plants. The fermentation was accompanied by a slow fall in *p*H from 6.4 to 5.4, during which gradual degradation of pectic substances from the mucilage was evidenced.

Bacterial populations of fermenting cherries exposed to air or in tap water were high compared with those encountered on beans fermenting under aseptic conditions. However, the process in either case was neither normal nor complete at the end of 70 hr, inasmuch as the parchment was sticky, the beans were discolored, and the fermentation was gassy with an obnoxious odor. In other words, whereas the normal surface flora of cherries brought about a normal fermentation, introduction of flora from air or water had an adverse effect on the process. The observation that the fermentation was not progressive in the case of surface-sterilized cherries kept under sterile water lent further support to the conclusion that cherry surface flora was, in fact, involved in the process, and that cherry enzymes were not active in the degradation of mucilage layer.

The pectinolytic ability displayed by the yeasts (Table 3) is clearly indicative of their role in the process. It must be mentioned that the strains grouped together as belonging to a particular species were essentially similar with respect to their growth characteristics and enzyme content, and therefore only their averages are presented in the table. The three *Saccharomyces* species were indeed highly pectinolytic in comparison with the *Schizosaccharomyces* species. Hence, the latter species was not studied further. Surprisingly, except for a solitary strain of *Flavobacterium*, none of the 22 bacterial isolates possessed any pectinolytic activity. In contrast to the observations of Vaughn et al. (14) and Frank, Lum, and Dela Cruz (3), coliform bacteria or *Erwinia* species were not encountered in these units. In the absence of other pectinolytic organisms and in the presence of large numbers of pectinolytic yeasts, it is reasonable to assume that the yeasts have a vital role in the degradation of mucilage layer. A parallel to this may be found in the results of Roelofsen (11), who succeeded not only in isolating pectinolytic yeasts from the fermenting cacao beans but in correlating the production of their pectic enzymes with the maceration of collenchyma tissue. However, he concluded that the fermentation of cacao may be the result of activity of several other organisms as well. It is possible that in coffee fermentation, also, other organisms may be involved.

Demucilaging studies. The results of inoculation experiments with pure cultures of yeasts are illustrated in Table 4. *S. marixanus* could no doubt demucilage the seeds, but judging from other criteria the fermentation cannot be regarded as fully satisfactory. Among the various other combinations of yeasts tried, the fermentation brought about by the mixture of the three *Saccharomyces* species was the nearest approach

TABLE 2. *Population changes occurring during the natural fermentation of coffee cherries*

Time	*p*H	Pectin decomposed	No. of organisms per ml $\times 10^3$			
			Nutrient agar		Glucose yeast-extract-agar	
			Yeasts	Bacteria	Yeasts	Bacteria
hr		%				
0	6.4	0	43	32	107	112
4	6.2	4.4	124	58	396	386
12	6.1	13.6	456	185	688	402
24	5.9	25.2	923	302	1,120	386
36	5.8	38.4	1,250	451	1,800	378
48	5.7	58.6	2,350	863	2,100	359
60	5.6	70.0	2,790	785	3,110	324
70	5.4	98.3	5,500	659	3,480	316

TABLE 3. *Pectinolytic activity of isolates from coffee fermentation*[a]

Isolate	Enzyme activity[b]					Pectin decomposed
	PG		PE	PTE	PATE	
	P[c]	PA[c]	P	P	PA	
						%
Saccharomyces marxianus (6)[d]	0.5	3.5	0.5	0	0	90.5
S. bayanus (4)	0.3	3.0	0.42	0	0	65.5
S. cerevisiae var. *ellipsoideus* (15)	0.5	2.2	0.45	0	0	25.3
Schizosaccharomyces sp. (6)	0.1	0.3	0.1	0	0	10.6
Flavobacterium sp. (1)	0.9	0.6	0.18	0.4	0.2	76.8

[a] Isolates identified as *Streptococcus* sp. (6), *Flavobacterium* sp. (3), *Pseudomonas* sp. (6), and *Proteus* sp. (7) were nonpectinolytic.

[b] PG measured as increase in reducing power in terms of milliliters of 0.5 N sodium thiosulfate. PE as milliliters of 0.02 N sodium hydroxide, and PTE/PATE as units of optical density at 230 to 235 mμ.

[c] P = pectin; PA = polygalacturonic acid.

[d] Numbers in parenthesis indicate number of strains.

TABLE 4. *Fermentation of coffee cherries by pure cultures of yeasts*

Sample no.	Yeasts tested	Criteria observed			Pectin decomposed
		External appearance of beans	Flavor of ferment	Disappearannce of stickiness	
					%
1	*Saccharomyces marxianus*	Grayish-brown	Pungent	Almost complete	86.4
2	*S. bayanus*	Cream to yellow	Vinegar-like	Partial	59.0
3	*S. cerevisiae* var. ellipsoideus	White till 48 hr, black after 70 hr	Cheesy	Nil	27.6
4	Mixture of 1 and 2	Yellowish-brown	Vinegar-like	Almost complete	70.8
5	Mixture of 2 and 3	Brownish-black	Vinegar-like	Nil	38.5
6	Mixture of 1 and 3	Grayish	Pungent	Partial	53.3
7	Mixture of 1, 2, and 3	Blackish	Vinegar-like	Complete	94.6
8	Natural process	Original color retained with brown tinge	Vinegar-like	Complete	98.2

TABLE 5. *Effect of pectic enzymes on coffee fermentation*

Enzymes	Criteria observed			Percentage of pectin decomposed at				
	External appearance of beans	Flavor of the ferment	Disappearance of stickiness	2 hr	4 hr	6 hr	8 hr	10 hr
Yeast enzymes	Whitish to cream yellow	Vinegar-like	Complete	20.5	46.3	78.4	95.0	96.2
Benefax	Brownish-black	Nil	Complete	23.3	48.5	69.6	94.5	98.0
Pectinase	No change	Nil	Partial	8.3	20.4	36.7	43.3	56.9
No added enzymes (natural process)	Original color retained with brown tinge	Vinegar-line	Slight	3.8	4.4	5.6	8.9	13.7

to the natural process, although it left signs of discoloration on the beans. It is likely that under the natural conditions other microorganisms contribute in making the overall fermentation run a desirable course.

The pooled yeast enzyme preparation from the three *Saccharomyces* species was indeed very effective in the sense that it brought about complete elimination of pectic substances within 7 to 8 hr at room temperature. In fact, this crude

pooled enzyme was comparable in activity to the commercial mold pectinases (Table 5). The resultant product, moreover, was not only normal in appearance but was free from any off flavors. In other words, use of yeast enzymes in the processing of coffee can expedite its curing without affecting in any way its color or general appearance. It is difficult to infer from the present series of experiments whether the use of enzyme has any effect on the aroma of the final product. This possibility, however, exists, in the light of the report by Frazier (4) that cacao produced by enzymatic fermentation was inferior in flavor to the resultant product of natural fermentation. At the same time, it is clear that controlled and mild enzymatic degradation would not only hasten the separation of the seeds from the pulp but would obviate tainting ("onion flavor") occurring occasionally under prolonged natural fermentations. Use of enzyme preparation on a large scale for the curing of coffee beans is contemplated during the next season.

In contrast to the results of Frank, Lum, and Dela Cruz (3), in the present study only yeasts were found to dominate and possess the pectinolytic properties. The reason for this discrepancy is not clear. It is possible that environmental and procedural conditions prevailing at Kona (Hawaii) differ from those obtaining at Chikmagalur (Mysore, India), and these possibly influence the selection of a particular group of organisms. It may even be that soil, which presumably contributes the organisms involved in the process (some evidence for this has been adduced for Kona coffee by Frank and Dela Cruz and for Chikamagalur coffee by the authors, *unpublished data*), harbors a different population, or that the variety of cherries used may be of different composition, thereby selecting one particular group of organisms. Significantly, both the studies have shown that the cherry surface is inhabited by a large number of pectinolytic organisms which take part in the process, and that the cherry enzyme is not responsible for mucilage-layer degradation.

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