Involvement of pectolytic micro-organisms in coffee fermentation

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Summary During the fermentation of *Coffea arabica* L., the most frequently found pectolytic bacteria were *Erwinia herbicola* and *Klebsiella pneumoniae*. These micro-organisms produce pectatelyase which is unable to depolymerize esterified pectins of mucilage without previous de-esterification. Furthermore, the optimal activities are observed at pH 8.5 whereas fermentation conditions are acidic (5.3–3.5). The major lactic acid bacteria, *Leuconostoc mesenteroides*, do not produce pectolytic enzymes. Only a *Lactobacillus brevis* strain, rarely isolated with a low frequency, shows a polygalacturonase activity compatible with fermentation conditions. Mucilage decomposition seems to be correlated to acidification and not to enzymatic pectolysis. Inoculation with pectolytic micro-organisms allows microbiological control of the fermentation but does not speed up the process. It would be preferable to use lactic acid bacteria so that the pH remained as close as possible to natural fermentation, where acidification is important. This practice would standardize the coffee fermentation microflora and therefore control the end product quality.

Keywords Coffee fermentation, mucilage degradation, pectinase, pectolytic bacteria, wet process.

Introduction

In a wet processing method, coffee cherries are depulped to remove the skin (exocarp) and the pulp (outer mesocarp) (Fig. 1). The beans are then submitted to a fermentation (dry/wet) or a soaking to degrade the mucilaginous layer (inner mesocarp) which constitutes an obstacle to drying (Rolz *et al*., 1982). Mucilage can also be eliminated using a chemical agent (NaOH) or enzymes (pectinases) but fermentation is the least expensive method. These treatments decompose the mucilaginous substrate which is mainly constituted of simple sugars and pectic substances (Coleman *et al*., 1955; Calle, 1962; Garcia *et al*., 1991). A textural change is observed and washing can finally eliminate this mucilage. It is generally assumed that these changes are because of a pectolysis by the combined action of microbial and/or endogenous coffee enzymes (Frank & De la Cruz, 1964; Van Pee & Castelein, 1972; Menchù & Rolz, 1973), though no biochemical proof of this depolymerization by the pectolytic microflora has ever been presented. Yeasts were detected (Frank *et al*., 1965; Agate & Bhat, 1966; Van Pee & Castelein, 1970) but research showed that they were not able to grow on a pectin containing medium (Van Pee & Castelein, 1970; Avallone *et al*., 2001). In contrast to cacao mucilage degradation (Sanchez *et al*., 1984, 1985), yeasts are not responsible for the pectolysis. Micro-organisms that can be isolated on pectin medium mainly seem to be either *Klebsiella pneumoniae* or *Erwinia herbicola*, not reported as strong pectolytic bacteria (Perombelon & Kelman, 1980). The only

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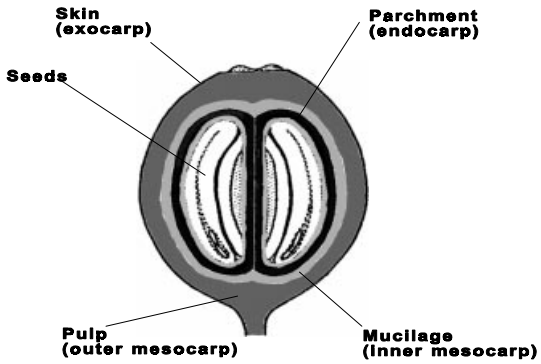

Figure 1 Longitudinal section of a coffee cherry according to Avallone *et al.* (2000b).

study of a pectolytic strain isolated from coffee fermentation, *E. dissolvens* (Frank & De la Cruz, 1964; Frank *et al.*, 1965), demonstrated that the pectate lyase produced was unable to depolymerize the highly methylated pectins of coffee mucilage (Castelein & Pilnik, 1976; Garcia *et al.*, 1991). Furthermore, a recent microscopical study has shown that the polysaccharides of mucilage cell walls were not completely degraded during fermentation (Avallone *et al.*, 1999). The involvement of pectolytic microbial enzymes (polygalacturonase, pectin lyase, pectate lyase and pectinmethylesterase) in the textural change of the mucilaginous layer is really controversial.

According to several authors, fermented coffee has a better quality than dry-processing coffee (Puerta-Quintero, 1999) but fermentation must be controlled to limit beverage defects (Gamble & Wootton, 1963; Woelore, 1993). When fermentation is incomplete, small remnants of intact mucilage are not eliminated by washing and can be consumed by micro-organisms in secondary fermentation during drying and storage (Gamble & Wootton, 1963; Woelore, 1993). Furthermore, over-fermentation (Lopez *et al.*, 1989) and bad fermentation, with butyric or propionic acids production (Wootton, 1961, 1965; Menchù & Rolz, 1973), would be, respectively, responsible for the 'alcoholic' and 'stinker' coffee tastes (Wootton, 1961; Gibson & Butty, 1975; Bade-Wegner *et al.*, 1997). Several authors have pointed out the fact that coffee fermentation must be controlled but few experiments have investigated the way to standardize the microflora. The only published data on microbiological control reported the use of pectolytic yeast inocula (Agate & Bhat, 1966) or residual waters from a previous fermentation (Calle, 1957, 1965; Butty, 1973).

The aim of the present investigation was to test the hypothesis that pectolytic micro-organisms could be isolated from the total microflora of coffee fermentation and their enzymatic activities characterized. The selected strains were then reintroduced as inocula in the coffee mass to see if fermentation could be microbiologically controlled and/or improved.

Materials and methods

Reagents

Culture media were purchased from Biokar (Biokar Diagnostics company, France). Polygalacturonic acid (PGA), apple pectin and other chemicals were from Sigma (St Louis, MO, USA).

Strains

Micro-organisms were obtained from a collection of 176 strains identified in a previous work on *Coffea arabica* L. fermentation microflora during the 1996–1997 season. These micro-organisms were isolated during fermentation where the pH decreased from 5.3 to 3.5 in 20 h (Avallone *et al.*, 2001). A pectolytic yeast from cocoa fermentation, *Kluyveromyces fragilis* K211 (Sanchez *et al.*, 1984; 1985), was also used as inoculum.

Culture conditions

Microbial batch cultures were grown at 30 °C in 50 mL flasks (Sakellaris *et al.*, 1989). Bacteria and yeasts were cultivated with a good aeration in a broth containing sodium polypectate (0.5%), casaminoacids (0.5%), Na_2HPO_4 (0.06%), KH_2PO_4 (0.21%) and $MgSO_4$ (0.02%) (Nassar *et al.*, 1994). Lactic acid bacteria were cultivated in a modified De Man Rogosa and Sharpe (MRS) broth supplemented with 0.1% apple pectin (w/v) (Karam & Belarbi, 1995). After incubation, cell suspensions were centrifuged at 4 °C (20 min, 11 500 ***g***) and supernatants were immediately frozen at –20 °C. Cells were washed twice with sodium acetate (0.1 M, pH 5.3) and centrifuged a second time. The pellet was harvested at –20 °C. Supernatants and cells

were then used to detect extracellular or cellular pectolytic activities.

Inocula of pectolytic strains were prepared for fermentation seeding from 24 h-old-cultures in the same broth. Cells were obtained by centrifugation (20 min, 11 500 ***g***) and washed twice with sodium acetate (0.05 M, pH 4.5) (Sakellaris *et al.*, 1989). After inoculation, micro-organisms were numbered on plates incubated during 48 h at 30 °C: lactic microflora on De Man Rogosa and Sharpe agar (MRS) (De Man *et al.*, 1960), yeast on Yeast Glucose Chloramphenicol agar (YGC) containing 0.25 g L^{-1} of the antibiotic and aerobic bacteria on Plate Count Agar (PCA). Micro-organisms able to grow with pectin as sole source of carbon were enumerated on Boccas medium pH 6.8 (Boccas *et al.*, 1994).

Viscosimetric assay

After incubation of each strain in the broth described above, the culture supernatants were used to detect endopectolytic enzymes by the viscosity reduction method (Sanchez *et al.*, 1984). Endoenzymes, like polygalacturonase (PG), pectin lyase (PL) and pectate lyase (PAL), have to cleave about 5–10% of glycosidic bounds to reduce the pectin solution viscosity by 50% (Juven *et al.*, 1985). Flow times were measured at 25 °C using an AVS 400 capillary viscometer (Schott Geräte, Hofheim, Germany) and compared with 0.1 M sodium acetate as reference.

Visualization of pectolytic activities on plates

An aliquot (50–100 μL) of either cell suspensions or culture supernatants were incubated on solid media (McKay, 1988; 1990). PG and PAL activities were visualized on plates containing PGA (0.5%), agar (2%), and, respectively, sodium acetate (0.1 M, pH 5.3) or Tris–HCl (0.05 M, pH 8.6). Pectinmethylesterase (PME) and PL activities were detected on plates containing esterified pectins from apple (0.5%), agar (2%) and citrate-phosphate (0.05 M, pH 6.5). Plates were then incubated during 2, 4 or 8 h at 37 °C. Halos of PME activity were revealed by 0.1 M malic acid during 1 h followed by 0.02% (w/v) ruthenium red solution during 12 h. Halos of PG and PAL activities were stained by 1% cetyl trimethyl ammonium bromide (w/v) during 12 h (Van Gijsegem, 1986). If substrate degradation had occurred around the supernatant culture or cells suspension, halos would surround the inoculation.

Concentration by ultrafiltration

Supernatant concentration was necessary to detect low enzymatic activities. The crude supernatant (15 mL) was concentrated to a final volume of 0.5 mL using ultrafiltration Ultra-Free systems (Millipore, Bedford, MA) with a 30 kDa cut-off membrane (Karam & Belarbi, 1995). The retentates were stored at –20 °C.

Enzymatic assays

Hydrolysis of glycosidic bounds by PG activity was followed by the end-reducing groups analysis (Somogyi, 1952). PAL assay was performed using a reaction mixture containing Tris–HCl (0.1 M, pH 8.6), 1 mM $CaCl_2$ and 0.5% PGA incubated at 30 °C (Moran *et al.*, 1968). The unsaturated compounds released from PGA by the PAL activity were spectrophotometrically measured at 235 nm. A unit of PAL activity (U) corresponds to the formation of 1 μmol unsaturated uronide liberated per minute. Assays were repeated three times and all controls were performed using a heat-denatured enzyme. To determine optimal pH activities, enzymatic assays were buffered with sodium acetate (0.1 M), for the range of pH from 3.5 to 6.0 and Tris–HCl (0.1 M) for the pH range from 6.0 to 11.0.

Inoculated fermentation

Coffee was depulped and fermented in dry conditions in order to limit the contamination of the coffee endogenous microflora by the micro-organisms of water. Inocula were added to coffee mass (~10 kg) at the rate of 0.2% (w/w) and stirred during 5 min. The temperature was maintained between 19 and 22 °C. Fermented beans (50 g) were sampled each 5 h to determine pH (Lopez *et al.*, 1989) and microbial counts (Avallone *et al.*, 2001).

Organoleptic tests

At the end of the process, roasted coffee was sampled, added to boiling water at the rate of 2% (w/v) and infused during 5 min. Drinks were

prepared and tasted in triangular tests. For each sample, the jury members (10 persons) evaluated acidity, astringency, coffee aroma, bitterness, aroma intensity, preference and overall impression (as defect). A note, from 0 to 10, was attributed to each criteria. Statistical analysis (Student's *t*-test) was used to look for significant organoleptic differences.

Results and discussion

Two strategies were used to select the pectolytic strains of coffee fermentation microflora: screening by viscosimetric assay using the total microflora and screening using the cup plates technique on the microflora able to grow with pectin as sole source of carbon.

Pectolytic micro-organisms screening by viscosimetric technique

The most frequent yeasts (19 strains) and lactic acid bacteria (33 strains) of coffee fermentation were tested by viscosimetric assays to detect endoenzymes (PG, PL and PAL) (Table 1). Yeast growth did not appear to have any effect on the viscosity of the culture medium. These micro-organisms did not produce endopectolytic enzymes in agreement with our previous observations on a medium containing pectin as sole source of carbon (Avallone *et al.*, 2001). The tested strains were effectively not pectolytic strains according to Luh & Phaff (1951) and Sanchez *et al.* (1985). The most frequently found lactic acid bacteria (*Leuconostoc mesenteroides*) did not produce endopectolytic enzymes contrary to the hypothesis of Juven *et al.* (1985). Only a *Lactobacillus brevis* L166 strain decreased the supernatant viscosity significantly (~45%) and was selected for further investigations.

This viscosimetric technique showed that the most common lactic acid bacteria and yeasts of coffee fermentation are not strong producers of endopectolytic enzymes (PG, PAL and PL) except *L. brevis* L166 which was rarely isolated.

Table 1 Endopectolytic activities of the most frequent yeasts and lactic acid bacteria of coffee fermentation determined by viscosimetric assay

Isolates	Flow times decrease*
Yeast	
Kloeckera apis apiculata L2	–
Kloeckera apis apiculata L9	–
Kloeckera apis apiculata L15	–
Kloeckera apis apiculata L23	–
Cryptococcus laurentii L13	–
Cryptococcus laurentii L19	–
Cryptococcus albidus L18	–
Candida lambica L4	–
Candida famata L11	–
Candida colliculosa L12	–
Candida guillermondii L20	–
Lactic acid bacteria	
Leuconostoc mesenteroides L121	–
Leuconostoc mesenteroides L128	–
Leuconostoc mesenteroides L129	–
Leuconostoc mesenteroides L134	–
Leuconostoc mesenteroides L135	–
Leuconostoc mesenteroides L142	–
Leuconostoc mesenteroides L145	–
Lactococcus lactis lactis L124	–
L. brevis L166	+ (~45%)

* – No significant flow times decrease; + significant flow times decrease.

Pectolytic micro-organisms screening by plates techniques

The most frequently isolated micro-organisms on a pectin-containing medium, *E. herbicola* and *K. pneumoniae*, were tested with the plate technique to characterize their enzymatic activity. However, it must be emphasized that enzymatic activities were not detected without previous concentration of the supernatant, confirming that the major pectolytic micro-organisms of coffee fermentation are not strong producers of pectolytic enzymes (Table 2).

In the concentrated supernatant, PME activity was always absent. This was surprising because a previous de-esterification of coffee mucilage pectins would be necessary to allow the PG or PAL activities. These results are in agreement with the observation of the micro-organisms on plates: colonies were small in pectin containing medium and largest when PGA was used as substrate. Pectolytic micro-organisms can easily consume PGA but not esterified pectins.

Several strains of *K. pneumoniae*, *E. herbicola* and *Bacillus subtilis* showed PAL activity. The halo diameters were always largest with *E. herbicola* than *K. pneumoniae*, suggesting a stronger enzymatic activity. *L. brevis* L166 produced a PG

Table 2 Enzymatic activities of the culture medium of the pectolytic microflora

	Pectolytic activities	
Isolates	**Supernatant**	**Concentrated supernatant**
Enterobacteriacae*		
E. herbicola C5	–	PAL
E. herbicola C9	–	PAL
E. herbicola C10	–	PAL
E. herbicola C15	–	PAL
E. herbicola C26	–	PAL
K. pneumoniae C1	–	PAL
K. pneumoniae C2	–	PAL
K. pneumoniae C7	–	PAL
K. pneumoniae C12	–	PAL
Gram + bacilli†		
B. subtilis C12	PAL	PAL
L. brevis L166	PG	PG

* Micro-organisms isolated with high frequency.
† Micro-organisms rarely isolated.
PAL, pectate lyase activity; PG, polygalacturonase activity.

activity as earlier identified for *Lactobacillus plantarum* (Sakellaris *et al*., 1988; 1989) and *Lactobacillus casei* (Karam & Belarbi, 1995). The enzymatic activities were always extracellular except the *L. brevis* L166 PG associated with the cells.

These two screening strategies allowed us to select the three pectolytic strains of coffee fermentation microflora: *E. herbicola* C26, *B. subtilis* C12 and *L. brevis* L166.

Effect of pH on pectolytic activities

The effect of pH on the pectolytic activities of the three selected strains was evaluated in order to see if these enzymes could be active during the fermentation (Fig. 2). The optimal PAL activities of *E. herbicola* C26 and *B. subtilis* C12 were, respectively, pH 9.0 and 8.2 in agreement with those observed by Nasser *et al.* (1990) and Chesson (1978) with *B. polymyxa* and *B. subtilis* (~8.5). These enzymes could not be active at the fermentation pH (5.3–3.5). The maximal activity of *L. brevis* L166 PG was at pH 4.2 whereas *L. plantarum* activity was best at pH 4.5 (Sakellaris *et al*., 1988; 1989). This PG would be the only enzyme that could be efficient at the fermentation pH although this strain was rarely isolated (Avallone *et al*., 2001).

These results allowed us to conclude that the most frequent pectolytic micro-organisms of coffee microflora (*E. herbicola* and *K. pneumoniae*) produce enzymatic activities unable to depolymerize mucilage pectin at the fermentation pH, except for *L. brevis* L166 which appears not to be important in coffee fermentation.

Inoculation and organoleptic tests

The microbial and physicochemical parameters of fermentation inoculated by pectolytic micro-organisms are summarized in Table 3. Inoculated micro-organisms survived in coffee mass but no growth was observed except when a lactic acid bacteria (*L. brevis* L166) was used. Contrary to a previous work (Butty, 1973), pectolytic inocula did not speed up the mucilage degradation and inoculated fermentation took the same time as the control. Inocula must produce all the pectolytic activities (PAL, PL, PG and PME) to significantly improve the mucilage degradation as suggested by Agate & Bhat (1966). It is difficult to say if the micro-organisms have really produced their enzymes or if these enzymes were inefficient on the substrate or at the fermentation pH.

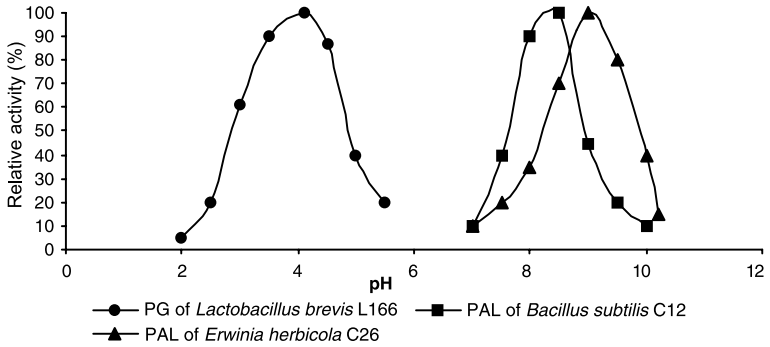

Figure 2 Optimal pH of the pectolytic activities of the selected strains.

Table 3 Characteristics of inoculated fermentation and coffee beverage

	Fermentation characteristics			Beverage characteristics	
	Survival*	Growth*	pH†	Acidity‡	Appreciation§
Reference			3.5	2.5	2.6
Lb. brevis L166	+	+	3.5	2.7	2.7
E. herbicola C26	+	−	4.1	2.1	2.2
B. subtilis C12	+	−	4.0	2.2	2.3
K. fragilis K211	+	−	4.4	2.1	2.1

* Estimation of the survival and growth of inoculated micro-organisms by microbial counts.
† Final pH compared with the reference after 20 h.
‡ Acidity note from 0 to 5 attributed to each beverage.
§ Higher score denotes better appreciation.

Mucilage decomposition took 2–3 days in sterile conditions (data not shown). Contrary to Rolz *et al.* (1971), endogenous coffee enzymes are insufficient to degrade the tissue.

Statistical analysis of tasting results showed that inoculation had no significant influence on coffee beverage quality. Organoleptic characteristics (acidity and preference) of coffee beverage were closest to the reference when lactic acid bacteria were used in the fermentation step. These kinds of micro-organisms degrade the simple sugars by lactic acid fermentation whereas yeast and enterobacteriaceae produce alcoholic or mixed acid fermentation. Organoleptic differences were not significant using a Student's *t*-test or Fischer analysis.

Conclusion

Previously (Avallone *et al.*, 2001), it was observed that the level of pectolytic microflora did not increase during the fermentation step. The present research has shown that these pectolytic strains, mainly *E. herbicola* and *K. pneumoniae*, produce PAL activities inefficient at the fermentation pH (5.3–3.5). These enzymatic activities cannot degrade highly esterified pectins. PME activity would be necessary to liberate PGA, the PAL substrate, but these esterases were absent in all the isolates. These results lead to the conclusion that depolymerization of pectic substances by pectolytic micro-organisms does not occur or is negligible during fermentation. Van Pee & Castelein (1972) have earlier demonstrated that *E. dissolvens*, a coffee fermenting micro-organisms, only produced a PAL unable to depolymerize highly methylated pectins of mucilage. These results are in agreement with the microscopical observations of the mucilage cell walls which have demonstrated that they were still rich in polysaccharides (pectins, cellulose, hemicellulose) after fermentation (Avallone *et al.*, 1999). These quantitative and qualitative results allow us to conclude that the mucilage dissolution is not because of a microbial pectolysis contrary to the general assertion (Calle, 1957; Frank & De La Cruz, 1964; Frank *et al.*, 1965; Agate & Bhat, 1966; Arunga, 1973).

As suggested by several authors, mucilage degradation seems to be correlated to acidification (Calle, 1965; Wootton, 1965). Microbial growth is necessary but the microflora does not directly participate in mucilage degradation by enzyme production. Its role is to produce metabolites such as organic acids (lactic and acetic acids) inducing a pH decrease (Calle, 1965; Arunga, 1973; Lopez *et al.*, 1989). As a consequence, the mucilage cell walls swelling capacity in water is modified as well as their bound calcium (data not shown). These alterations loosen the polysaccharide network with a clear textural change. These mechanisms are well known in the cellular response to microbial attack observed in plant/pathogen interactions (D'Auzac, 1996). Nevertheless, the involvement of the microflora on the aroma and acidity of coffee beverage has to be studied.

Inoculations confirmed that pectolytic micro-organisms do not speed up polysaccharide degradation. Organoleptic characteristics of the beverage were not modified. The microbial control of coffee

fermentation microflora would be possible in order to limit off-flavour development and to standardize the final coffee quality. It would be preferable to use lactic acid bacteria in order to stay as close as possible to the natural fermentation. Mixed inocula of lactic acid bacteria (*L. brevis* L166, *L. casei* or *L. plantarum*) with various pectolytic activities (PME, PL, PG, PAL) would probably improve polysaccharide degradation and perhaps limit the secondary fermentation observed during drying and storage.

References

Agate, A.D. & Bhat, J.V. (1966). Role of the pectolytic yeasts in the degradation of mucilage layer of *Coffea robusta* cherries. *Applied Microbiology*, **14**, 256–260.

Arunga, R.O. (1973). Enzymatic fermentation of coffee. *Kenya Coffee*, **38**, 354–357.

Avallone, S., Guyot, B., Michaux-Ferrière, N. *et al*. (1999). Cell wall polysaccharides of coffee bean mucilage. Histological characterization during fermentation. In: *Association Scientifique Internationale Du Café* (edited by ASIC). Pp. 463–470. 18th International Scientific Colloqium on Coffee, Helsinki. Paris: Association Scientifique Internationale du Café (ASIC).

Avallone, S., Guiraud, J.P., Guyot, B., Olguin, E. & Brillouet, J.M. (2000). Polysaccharide constitution of coffee bean mucilage. *Journal of Food Science*, **65**, 1308–1311.

Avallone, S., Brillouet, J.M., Guyot, B., Olguin, E. & Guiraud, J.P. (2001). Microbiological and biochemical study of coffee fermentation. *Current Microbiology*, **42**, 252–256.

Bade-Wegner, H., Bendig, I., Holscher, W. & Wollmann, R. (1997). Volatile compounds associated with the overfermented flavour defect. In: *Association Scientifique Internationale Du Café* (edited by ASIC). Pp. 176–182. 17th International Scientific Colloqium on Coffee, Nairobi. Paris: Association Scientifique Internationale du Café (ASIC).

Boccas, F., Roussos, S., Gutierrez, M., Serrano, L. & Viniegra, G.G. (1994). Production of pectinase from coffee pulp in solid state fermentation system: selection of wild fungal isolate of high potency by a simple three-step screening technique. *Journal of Food Science and Technology*, **31**, 22–26.

Butty, M. (1973). Rapid fermentation of coffee. *Kenya Coffee*, **38**, 214–224.

Calle, V.H. (1957). Activadores bioquimicos para la fermentation del café. *Cenicafé (Colombia)*, **8**, 94–101.

Calle, V.H. (1962). Métodos de extraccion de las pectinas del café. *Cenicafé (Colombia)*, **13**, 69–74.

Calle, V.H. (1965). Algunos metodos de desmucilaginado y sus efectos sobre el café en pergamino. *Cenicafé (Colombia)*, **16**, 3–11.

Castelein, J.M. & Pilnik, W. (1976). The properties of the pectatelyase produced by *Erwinia dissolvens*, a coffee fermenting organism. *Lebensmittel Wissenschaft und Technologie*, **9**, 277–283.

Chesson, L. (1978). The maceration of vegetable tissue by a strain of *Bacillus subtilis*. *Journal of Applied Bacteriology*, **44**, 347–367.

Coleman, R.J., Lenney, J.F., Coscia, A.T. & Dicarlo, F.J. (1955). Pectic acid from the mucilage of coffee cherries. *Archives of Biochemistry and Biophysics*, **59**, 157–164.

D'Auzac, J. (1996). 'Toxic' oxygen: protection against pathogens. *Plantations, Recherche, Développement*, **3**, 153–170.

De Man, J.C., Rogosa, M. & Sharpe, M.E. (1960). A medium for the cultivation of lactobacilli. *Journal of Applied Bacteriology*, **23**, 130–135.

Frank, H.A. & De la Cruz, A.S. (1964). Role of incidental microflora in natural decomposition of mucilage layer in Kona coffee cherries. *Journal of Food Science*, **29**, 850–853.

Frank, H.A., Lum, A.N. & De la Cruz, A.S. (1965). Bacteria responsible for mucilage layer decomposition in Kona coffee cherries. *Applied Microbiology*, **13**, 201–207.

Gamble, F.M. & Wootton, A.E. (1963). A note on a very slow fermentations. *Kenya Coffee*, **28**, 481–483.

Garcia, R., Arriola, D., Arriola, M.C., Porrez, E. & Rolz, C. (1991). Characterization of coffee pectin. *Lebensmittel Wissenschaft und Technologie*, **24**, 125–129.

Gibson, A. & Butty, M. (1975). Overfermented coffee beans: a method for their detection and elimination. In: *Association Scientifique Internationale Du Café* (edited by ASIC). Pp. 141–152. 7th International Scientific Colloqium on Coffee, Hamburg. Paris: Association Scientifique Internationale du Café (ASIC).

Juven, B.J., Lindner, P. & Weisslowicz, H. (1985). Pectin degradation in plant material by *Leuconostoc mesenteroides*. *Journal of Applied Bacteriology*, **58**, 533–538.

Karam, N.E. & Belarbi, A. (1995). Detection of polygalacturonases and pectin esterases in lactic acid bacteria. *World Journal of Microbiology and Biotechnology*, **11**, 559–563.

Lopez, C.I., Bautista, E., Moreno, E. & Dentan, E. (1989). Factors related to the formation of 'overfermented coffee beans' during the wet processing method and storage of coffee. In: *Association Scientifique Internationale Du Café* (edited by ASIC). Pp. 373–384. 13th International Scientific Colloqium on Coffee, Paipa. Paris: Association Scientifique Internationale du Café (ASIC).

Luh, B.S. & Phaff, H.J. (1951). Studies on polygalacturonase of certain yeasts. *Archives of Biochemistry and Biophysics*, **33**, 212–227.

McKay, A.M. (1988). A plate assay method for the detection of fungal polygalacturonase secretion. *FEMS Microbiology Letters*, **56**, 355–358.

McKay, A.M. (1990). Degradation of polygalacturonic acid by *Saccharomyces cerevisiae*. *Letters in Applied Microbiology*, **11**, 41–44.

Menchù, J.F. & Rolz, C. (1973). Coffee fermentation technology. *Thé Café Cacao*, **17**, 53–61.

Moran, F., Nasuno, S. & Star, M.P. (1968). Extracellular and intracellular polygalacturonic acid trans-eliminase of *Erwinia carotovora*. *Archives of Biochemistry and Biophysics*, **123**, 298–306.

Nassar, A., Bertheau, Y., Dervin, C., Narcy, J.P. & Lemattre, M. (1994). Ribotyping of *Erwinia chrysanthemi* strains in relation to their pathogenic and geographic distribution. *Applied and Environmental Microbiology*, **60**, 3781–3789.

Nasser, W., Chalet, F. & Robert-Baudouy, J. (1990). Purification and characterization of extracellular pectate-lyase from *Bacillus subtilis*. *Biochimie*, **72**, 689–695.

Perombelon, M.C.M. & Kelman, A. (1980). Ecology of the soft-rot erwinia. *Annual Review of Phytopathology*, **18**, 361–387.

Puerta-Quintero, G.I. (1999). Influencia del proceso de beneficio en la calidad del cafe. *Cenicafé (Colombia)*, **50**, 78–88.

Rolz, C., Menchù, J.F., Espinosa, R. & Garcia-Prendes, A. (1971). Coffee fermentation studies. In: *Association Scientifique Internationale Du Café* (edited by ASIC). Pp. 259–268. 5th International Scientific Colloqium on Coffee, Lisbon. Paris: Association Scientifique Internationale du Café (ASIC).

Rolz, C., Menchù, J.F., Calzada, F. & De Leon, R. (1982). Biotechnology in washed coffee processing. *Process Biochemistry*, **17**, 8–10.

Sakellaris, G., Nikolaropoulos, S. & Evangelopoulos, A.E. (1988). Polygalacturonase biosynthesis by *Lactobacillus plantarum*: effect of cultural conditions on enzyme production. *Journal of Applied Bacteriology*, **65**, 397–404.

Sakellaris, G., Nikolaropoulos, S. & Evangelopoulos, A.E. (1989). Purification and characterization of an extracellular polygalacturonase from *Lactobacillus plantarum* strain BA 11. *Journal of Applied Bacteriology*, **67**, 77–85.

Sanchez, J., Daguenet, G., Guiraud, J.P., Vincent, J.C. & Galzy, P. (1985). A study of the yeast flora and the effect of pure culture seeding during the fermentation process of cocoa beans. *Lebensmittel Wissenschaft und Technologie*, **18**, 69–75.

Sanchez, J., Guiraud, J.P. & Galzy, P. (1984). A study of the polygalacturonase activity of several yeast strains isolated from cocoa. *Applied Microbiology and Biotechnology*, **20**, 202–207.

Somogyi, M. (1952). Notes on sugar determination. *Journal of Biological Chemistry*, **195**, 19–23.

Van Gijsegem, F. (1986). Analysis of pectin-degrading enzymes secreted by three strains of *Erwinia chrysanthemi*. *Journal of General Microbiology*, **132**, 617–624.

Van Pee, W. & Castelein, J.M. (1970). The yeast flora of fermenting robusta coffee. *East African Agricultural Journal*, **36**, 308–311.

Van Pee, W. & Castelein, J.M. (1972). Study of the pectinolytic microflora, particularly the Enterobacteriaceae, from fermenting coffee in the Congo. *Journal of Food Science*, **37**, 171–174.

Woelore, W.M. (1993). Optimum fermentation protocols for Arabica coffee under Ethiopian conditions. In: *Association Scientifique Internationale Du Café* (edited by ASIC). Pp. 727–733. 15th International Scientific Colloqium on Coffee, Montpellier. Paris: Association Scientifique Internationale du Café (ASIC).

Wootton, A.E. (1961). Fermentation and onion flavour. *Kenya Coffee*, **26**, 126–129.

Wootton, A.E. (1965). The importance of field processing to the quality of east african coffees. In: *Association Scientifique Internationale Du Café* (edited by ASIC). Pp. 247–254. 2th International Scientific Colloqium on Coffee, Nairobi. Paris: Association Scientifique Internationale du Café (ASIC).

