Domination among Strains of Lactic Streptococci with Attention to Antibiotic Production

E. B. Collins

Department of Food Science and Technology, University of California, Davis, California

Received for publication July 28, 1960

When different strains of lactic streptococci are mixed and propagated for use in manufacturing cultured dairy products, one strain will often dominate and become responsible for producing most of the lactic acid (Nichols and Ineson, 1947; Czulak and Hammond, 1954; Collins, 1955; Lightbody and Meanwell, 1955). The domination may develop in only one or two daily propagations, or it may develop slowly and become pronounced only after the mixtures are propagated for several days or weeks, depending on the cultures mixed. When strain domination has occurred, a single bacteriophage active against the dominant strain can cause failure in the production of lactic acid.

Hoyle and Nichols (1948) found that some antibiotic-producing cultures of lactic streptococci dominated some cultures that did not produce antibiotics. Lightbody and Meanwell (1955) studied domination among several strains of lactic streptococci and concluded that rapid domination was the result of the production of antibiotics. They suggested that slow domination might result from small undetected amounts of antibiotics or, alternatively, from small differences in growth rate. Collins (1955) failed to find antibiotics produced by any of five cultures among which domination occurred, but found small differences in acid tolerance.

Czulak and Hammond (1954) reported that blends of *Streptococcus lactis* and *Streptococcus cremoris* were more stable than blends of two *S. cremoris* strains. Investigations of these bacteria by Lightbody and Meanwell (1955) and Collins (1955) indicated on the contrary that there is no relationship between species and ability to dominate.

The present work was undertaken to study domination among 33 strains of lactic streptococci, most of them used extensively in making cheese (Whitehead, 1953; Czulak and Naylor, 1956; Collins, 1958). The results help to clarify the influences of antibiotic production and of differences in competitive growth ability. They also clarify the sensitivities of different species and strains of lactic streptococci to the antibiotics produced.

Materials and Methods

Cultures. Table 1 lists the 33 cultures of lactic streptococci used, and their sources and bacteriophage sensitivities. There were 22 cultures of *Streptococcus cremoris*, 5 of *Streptococcus lactis*, and 6 of *Streptococcus diacetilactis* (Swartling, 1951). Differentiation of the species was based on the following reactions: type of action on litmus milk at 22 C, production of ammonia from arginine, growth in litmus milk at 40 and 45 C, growth in broth containing 4 per cent and 6.5 per cent sodium chloride, fermentation of maltose, fermentation of dextrin, production of acetoin, and production of gas.

Propagating cultures. Cultures were propagated in tubes of sterile skim milk fortified with 0.75 per cent nonfat milk solids. The skim milk was examined for inhibitors (Collins, 1957); any containing inhibitors was not used. In propagating cultures, tubes of fortified skim milk were inoculated with 1.5 per cent culture, incubated 16 hr at 22 C, and refrigerated until repropagated. Cultures were started from the lyophilized state and propagated once daily for 3 to 7 days before they were mixed for determining compatability.

Determining antibiotic production. Each culture was tested for production of antibiotics and for sensitivity to the antibiotics produced, by the following procedure: tryptone yeast phosphate agar (Hunter, 1946) was poured into Petri plates and permitted to solidify. The plates of agar, open and inverted, were dried at 37 C until the agar surfaces were very slightly wrinkled. They were then cooled to room temperature, and one or two drops of each culture to be tested for sensitivity were smeared evenly over an agar surface with a bent glass rod. Cultures to be tested for the production of antibiotics were grown for 24 hr at 22 C. One drop of each was placed on an agar surface smeared with a test culture. The inoculated plates were incubated right-side up at 22 C and observed after 24 and 48 hr for clear zones of inhibition around the drops of cultures being tested.

Determining strain compatability. Mixtures of two cultures were prepared by inoculating one drop of each into a tube of fortified skim milk. Mixtures were incubated and subsequently propagated daily (6 days per week).

The strains of bacteria in each mixture were tested for domination, usually after 1, 7, and 14 daily propagations. Four tubes of litmus skim milk were inoculated with a mixture, one drop per tube. One tube served as a control, indicating activity of the mixture. The second

tube received three drops of each of the two corresponding bacteriophages and served as a check on the bacteriophage sensitivities of the cultures. The third tube received three drops of one bacteriophage and the fourth tube three drops of the other. The four tubes of inoculated skim milk were compared after incubation for 7 hr at 32 C. This was about 1 hr after the controls had coagulated. From the last two tubes it could be determined whether one of the strains of bacteria had dominated. When one bacteriophage retarded coagulation of the mixture, the corresponding strain of bacteria was considered predominant. Three gradations of retardation, *i.e.*, dominance, were determined as follows. Test cultures that were reduced and weakly coagulated, reduced and not coagulated, and not reduced or coagulated were considered to indicate very slight, slight, and marked domination, respectively.

TABLE 1

Description of the single-strain cultures of the lactic group of Streptococcus studied

Streptococcus Species	Culture	Source	Bacteriophage Sensitivity
S. cremoris	KH	New Zealand	kh
	C11	Australia	c11
	BR4	New Zealand	br4
	HP	Australia	hp
	KHR	Culture KH	khr
	FC4	Comm. culture	fc4
	Z	Comm. culture	z
	C13	New Zealand	c13
	K2	Comm. culture	e8
	LT8	Comm. culture	1t8
	R1	Australia	r1
	ASF	Comm. culture	Resistant*
	C7	Australia	c7
	4B	Comm. culture	4b
	15C	Comm. culture	15c
	AB3	Comm. culture	ab3
	H6	Comm. culture	h6
	C1	Australia	c1,20a
	20A	Raw milk	c1,20a
	C3	Australia	c3, m1 4
	ML4	New Zealand	c3, m1 4
	990	England	Resistant*
S. lactis	C10	Australia	c10
	C2	Australia	c2
	C6	Australia	c6
	356	England	Resistant*
	365	England	Resistant*
S. diacetilactis	DRC1	Australia	drc1
	957	England	drc1
	DRC2	Australia	drc2
	DRC3	Australia	drc3
	NZ	New Zealand	drc1, drc2, drc3
	910	Comm. culture	910

* This culture was resistant to each of the available bacteriophages.

A modification of the above test was used to retest compatability in cases where the test indicated domination among nonantibiotic-producing cultures that had been mixed and propagated only once. Each of the four tubes of litmus milk was inoculated with one drop of each of the two cultures, bacteriophages were added as described above, and the inoculated tubes of milk were incubated. An absence of apparent domination in this immediate test was considered to validate the previously obtained results indicating that one of the cultures dominated the other during one propagation.

Results

Cultural and bacteriophage sensitivity characteristics of the S. diacetilactis cultures. Each of the *S. diacetilactis* cultures included in this study coagulated litmus milk during overnight incubation at 22 C, grew at 40 C, grew in broth containing 4 per cent sodium chloride, produced gas, and produced acetoin detectable by the Voges-Proskauer test. None of them grew at 45 C or in 6.5 per cent sodium chloride. All but strain NZ fermented maltose and dextrin. Only strain DRC3 produced ammonia from arginine. None was sensitive to any of 23 bacteriophages active against cultures of *S. cremoris* and *S. lactis*, and none of the *S. diacetilactis* bacteriophages acted on the *S. lactis* or *S. cremoris* cultures.

Production of antibiotics. The described method indicated that 10 of the 33 cultures produced antibiotics (table 2). Five of the cultures not found to produce antibiotics (C13, C11, FC4, BR4, and 15C) were studied in further attempts to demonstrate antibiotic production. Cultures H6, 4B, C10, and C6 were selected and used as test cultures. The following modifications of the described method failed to indicate antibiotic production: (a) Cultures were incubated for 48 hr instead of 24 hr before drops of them were placed on agar surfaces smeared with test cultures. (b) Cultures were tested immediately after they had been grown for 5 hr at 32 C. (c) Cultures were centrifuged and drops of whey were used instead of coagulated culture. (d) Plates of agar were dried at 37 C for periods longer and shorter than the usual time. (e) The pH of the agar was decreased to pH 6.0 and to pH 5.3. The neutralized whey method of Lightbody and Menawell (1955) was also used. Neutralized wheys prepared from each of cultures C13, C11, FC4, and 15C had no greater inhibitory effect on the test cultures than wheys prepared from the test cultures themselves, even though the amount of whey in the whey-skim milk mixtures was increased to 75 per cent.

Strain domination by the cultures that produced antibiotics. For studying strain compatibility, cultures were divided into those that produced antibiotics and those that did not. Five cultures that produced antibiotics (cultures C1, 20A, C3, DRC1, and 910) were tested

for ability to dominate each of 22 cultures that did not produce antibiotics. (The NZ strain of *S. diacetilactis* was not included.)

Tests for domination after the 110 mixtures had been grown for only one 17-hr propagation revealed marked domination in 89 mixtures. Tests run after the remaining 21 mixtures had been propagated twice revealed marked domination in 18 of them. In each of these 107 cases the dominant culture was the one that produced antibiotics.

Two of the 107 and the three exceptional cases involved culture C13. This unusual culture did not produce antibiotics and had been found resistant to them, but these strain compatability experiments suggested variability in its resistance. It was suppressed in two propagations by cultures C3 and 20A. In the above experiment it suppressed DRC1 and 910 slightly, and C1 definitely, during 14 propagations. In a later experiment C13 was suppressed in two propagations by DRC1, 910, and C1.

Strain domination among cultures that produced antibiotics. Study of strain compatibility among cultures that produced antibiotics was limited by the unavailability of bacteriophages active against cultures 990, 356, and 365, and by the fact that the other seven cultures were only four types, as judged by bacteriophage sensitivity. Results for eight mixtures that were made and tested were as follows. Culture 20A definitely suppressed C3 in 14 propagations and slightly suppressed DRC1 and 910. Culture C3 grew compatibly with C1 and very slightly suppressed DRC1 and 910. Cultures DRC1 and 910 very slightly suppressed C1.

Strain domination among cultures that did not produce antibiotics. The 20 cultures of *S. cremoris* and *S. lactis* that did not produce antibiotics were tested for compatibility in all possible combinations of two. Tests after one propagation indicated slight domination in 25 mixtures and very slight domination in 18. After seven propagations there was marked domination in 44 mixtures, slight domination in 57, and very slight domination in 19. After 14 propagations there was marked domination in 86, slight domination in 54, and very slight domination in 13 mixtures. Table 3 gives the results after 14 propagations, listing the 20 cultures in order of ability to dominate.

The incubation time at 22 C was usually 16 hr, but initially 51 mixtures were prepared in duplicate and 24 hr was used routinely as the incubation time for one set of the cultures. Results with the different incubation times were the same, except as follows. Domination occurred somewhat sooner in five mixtures and somewhat later in four mixtures with the 24-hr incubation time.

Mixtures were usually tested for domination by incubation for 7 hr at 32 C, after addition of the appropriate bacteriophages. Fifteen mixtures, in which various degrees of suppression had been indicated by testing at 32 C, were also tested by incubation for 16 hr at 22 C. Results at 22 C indicated that suppression had occurred in all cases, but in 13 of the 15 cases the suppression was slightly less than that indicated in tests at 32 C.

Three of the bacteriophages used (br4, c2, and hp) were observed to have "nascent" action (Collins, 1952) on certain cultures. In such mixtures inhibition of the heterologous culture prevented determination of domination except in mixtures in which the heterologous culture dominated.

Influence of skim milk enrichment on occurrence of strain domination. The cultures with least ability to

TABLE 2

Inhibitory effects on lactic streptococci of antibiotics produced by different species of lactic group of Streptococcus

Test Culture	No. Similar Cultures	Action of Antibiotic Produced by:									
		S. cremoris strain:					*S. diacetilactis*, strain:			*S. lactis*, strain:	
		20A	C1	ML4	C3	990	DRC1	910	957	356	365
S. cremoris 4B	16*	+†	+	+	+	+	+	+	+	+	+
S. lactis C2	3*	+	+	+	+	+	+	+	+	+	+
S. diacetilactis DRC2	2*	+	+	+	+	+	+	+	+	+	+
S. diacetilactis DRC3	1‡	+	+	+	+	−	+	+	−	+	+
S. cremoris C1	6§	−	−	−	−	−	−	−	−	+	+
S. diacetilactis DRC1	3¶	−	−	−	−	−	−	−	−	+	+
S. lactis 356	2¶	+	+	+	+	+	+	+	+	−	−

* This was all of the cultures of this species studied except those listed below.

† + = Inhibition; − = inhibition not detected.

‡ Results indicating no inhibition of DRC3 by 990 and 957 were considered due, at least in part, to the fact that zones of inhibition with culture DRC3 were cloudy and difficult to detect. It also is possible that cultures 990 and 957 produced a comparatively low concentration of antibiotic.

§ In addition to the five *S. cremoris* cultures that produced antibiotic, this group includes *S. cremoris* C13.

¶ Antibiotic-producing cultures.

dominate were generally least active in the production of lactic acid. This observation led to comparison of domination in skim milk fortified with 0.75 per cent nonfat milk solids with domination in skim milk enriched with 0.75 per cent nonfat milk solids 0.5 per cent glucose, 0.5 per cent yeast extract, 0.5 per cent trypticase, and 0.5 per cent peptone. Sixteen mixtures were prepared and propagated simultaneously in the two media for study of the domination of cultures H6, 4B, C10, and C6 by cultures C13, C11, FC4, and 15C. Tests for domination were run after the mixtures had been propagated 1, 2, 4, 8, and 14 times. With several mixtures, results were the same in the two media. The differences observed were as follows: (a) Each of cultures C13, C11, FC4, and 15C slightly dominated culture C10 and markedly dominated C6 in 14 propagations or less in the fortified skim milk, but none of them dominated C10 or C6 in the enriched skim milk. (b) Culture 15C markedly dominated H6 in 14 propagations in the fortified skim milk, but not at all in the enriched skim milk. (c) In fortified skim milk, culture C13 required 8 propagations for marked domination of culture 4B, and culture C11 required 14 propagations, but in the enriched skim milk the respective number of propagations required for marked domination were only 2 and 8.

Study of the reactions of the cultures to various nutrients was not complete, but the following results indicate that some of the above findings may be attributed to differences in growth-stimulating effect. Each of the eight cultures was grown for 5½ hr at 30 C in duplicate in each type of skim milk, and increases in titratable acidity were determined. The difference, indicating stimulation attributable to skim milk enrichment, was determined for each culture by subtracting the increase in fortified skim milk from the increase in enriched skim milk. The differences, expressed as per cent titratable acidity, were: C13 = 0.14, C11 = 0.22, FC4 = 0.25, 15C = 0.22, H6 = 0.25, 4B = 0.13, C10 = 0.23, and C6 = 0.23.

Discussion

The results substantiate the conclusion of Lightbody and Meanwell (1955) that domination in one or two daily propagations among strains of lactic streptococci is usually due to the production of antibiotics by the cultures that dominate. The results do not substantiate their suggestion that slower domination is the result of the production of undetectable amounts of antibiotic. This possibility is refuted by the showing that cultures not found to produce antibiotics can be arranged in a specific order determined by ability to dominate, that in some cases the occurrence of domination was altered by the use of enriched skim milk as growth medium, and that domination occurred among cultures that apparently produced the same antibiotic. The results indicate that many cultures of lactic streptococci do not produce antibiotics and that domination among them, as well as among cultures that produce the same antibiotic, should be attributed to differences in competitive growth ability. This may be influenced by such factors as small differences in lag phase, growth rate, tolerance to fermentation end products, and/or nutritional requirements. Differences in response and, hence,

TABLE 3

Nonantibiotic-producing cultures of Streptococcus cremoris and Streptococcus lactis arranged according to ability to dominate

Culture	Suppressed:*	Was Suppressed by:
C13 (A)	$B^bC^aDFG^bHJKLMN^bOPRSTW$	None
C11 (B)	$C^aD^bE^bF^bGI^aJKLMN^bO^bPRSW$	A
FC4 (C)	$D^aFG^aJKL^bMN^bOP^bRSTW$	AB
BR4 (D)	$EJ^bLM^bO^bP^bRSTW$	AB
15C (E)	$F^bG^bHI^bJ^bK^bLMN^bOP^bRSTW$	BD
Z (F)	$H^bI^aJ^bKLMN^bO^bPRSTW$	ABCE
HP (G)	$H^bI^aJ^bK^aL^aMN^bO^bRSTW$	ABCE
ASF (H)	Not determinable	AEFG
KHR (I)	$JKL^bMN^bO^aPRSTW$	BCEFG
K2 (J)	$K^bL^bM^aO^bP^bR^bS^bT^bW^b$	ABCDEFGI
RI (K)	O^bP^aRSTW	ABCEFGIJ
AB3 (L)	M^bPRS^bTW	ABCDEFGIJ
LT8 (M)	$O^bP^bRS^bT^bW$	ABCDEFGIJL
KH (N)	RS^bTW	ABCEFGI
H6 (O)	P^bRT^bW	ABCDEFGIJKM
C7 (P)	RS^bTW	ABCDEFIJKLMO
4B (R)	S^bTW^b	ABCDEFGIJKLMNOP
C10 (S)	T^aW	ABCDEFGIJKLMNPR
C2 (T)	W	ACDEFGIJKLMNOPRS
C6 (W)	None	ABCDEFGIJKLMNOPRST

* The suppression was marked in 2 weeks or less, except as follows: a = very slight suppression; b = slight suppression.

the competitive ability of lactic streptococci undoubtedly may be influenced by differences in the physiological state of the microorganisms at the time of testing and by differences in the composition of the milk used as the growth medium.

Experiments to determine domination among the cultures that did not produce antibiotics were terminated after the mixtures had been propagated 14 times and tested. The results substantiate the conclusion that it is rather uncommon for strains of rapid acid-producing lactic streptococci to grow together for long periods in fixed proportion (Czulak and Hammond, 1954; Lightbody and Meanwell, 1955). Had the mixtures been propagated longer, it is probable that each culture would eventually have dominated all the cultures listed below it in the domination order. But, on the other hand, the present results show that, with proper culture selection, mixtures in which the strains of bacteria will grow compatibly for at least 2 weeks can be prepared.

Judged by the mutual inhibition results of the antibiotic-producing cultures, the 10 cultures apparently produced only two different antibiotics. The antibiotic produced by five cultures of *S. cremoris* and three cultures of *S. diacetilacuis* is possibly diplococcin (Oxford, 1944). The different antibiotic produced by the two *S. lactis* cultures is possibly nisin (Mattick and Hirsch, 1947).

Hirsch (1952) concluded that strains of *S. lactis* and *S. cremoris* produce distinct antibiotics, each directed primarily against the other. The *S. diacetilactis* cultures of this study, like those studied by Swartling (1951), resembled *S. lactis* in their reactions to certain tests. Specifically, the antibiotic-producing strains of this species reacted the same as *S. lactis* to cultural tests used for distinguishing between *S. lactis* and *S. cremoris*, except that they did not produce ammonia from arginine. (Toward the end of the study a DRC1 isolate that hydrolyzed arginine was found.) From the conclusion of Hirsch, based primarily on studies with antibiotic-producing strains of *S. cremoris* and *S. lactis* (Mattick and Hirsch, 1947; Hirsch and Grinsted, 1951), *S. diacetilactis* cultures would be expected to produce antibiotics similar to those produced by *S. lactis*. But three (and the ammonia-producing DRC1 isolate) produced an antibiotic that appeared to be the same as that produced by five cultures of *S. cremoris*. These results indicate that there is not a known species difference that determines the type of antibiotic produced.

There is no relationship between species and antibiotic sensitivity pattern, and antibiotic-producing strains of *S. cremoris* and *S. lactis* obviously do not produce antibiotics that are directed in the case of either species primarily against the other. This is shown by the antibiotic sensitivity patterns for the nonantibiotic-producing strains. Those for 16 cultures of *S. cremoris*, 3 cultures of *S. lactis*, and at least 2 cultures of *S. diacetilactis* were identical, regardless of species. The mutual inhibitions of antibiotic-producing strains observed by Mattick and Hirsch (1947) and Hirsch and Grinsted (1951), and those observed in the present study, apparently indicate, in addition to the fact that diplococcin and nisin are different, only that a given antibiotic-producing strain of lactic streptococci is resistant to the particular type of antibiotic produced by itself, and that this resistance does not constitute protection against antibiotics that are of different type.

Acknowledgments

Appreciation is expressed for the cultures and bacteriophages contributed by Dr. H. R. Whitehead, of the Dairy Research Institute, Palmerston North, New Zealand, by Mr. J. Czulak, of the Commonwealth Scientific and Industrial Research Organization, Highett, Victoria, Australia, and by Mr. L. J. Meanwell, of the United Dairies Research Laboratories, London, England.

The work reported on testing cultures for antibiotic production was completed at the Dairy Research Institute, where the author worked as a Fulbright Grantee.

Summary

Twenty-two cultures of *Streptococcus cremoris*, five of *Streptococcus lactis*, and six of *Streptococcus diacetilactis* were tested for production of antibiotics and used in preparing 308 different mixtures for a study of strain domination. Mixtures were propagated daily in sterile skim milk fortified with 0.75 per cent nonfat milk solids and tested by the addition of appropriate bacteriophages.

Five *S. cremoris* cultures and three *S. diacetilactis* cultures produced one antibiotic, as judged from the mutual inhibitions of antibiotic-producing cultures and the antibiotic sensitivities of nonantibiotic-producing strains. Two *S. lactis* cultures produced a different antibiotic. There was no relationship between known species differences and type of antibiotic produced or antibiotic sensitivity. Antibiotic resistance was rare, except that each antibiotic-producing culture was resistant to the particular type of antibiotic produced by itself.

In 107 of 110 mixtures, antibiotic-producing cultures markedly dominated nonantibiotic-producing cultures in only 1 or 2 days. These rapid dominations undoubtedly resulted from action of the antibiotics. In 153 of 190 mixtures involving only nonantibiotic-producing cultures, and in 7 of 8 mixtures involving only cultures that produced one type of antibiotic, slight to marked domination occurred in 2 weeks or less. Results indicated that this more gradual type of strain

domination resulted from differences in the competitive growth ability of the cultures involved.

REFERENCES

COLLINS, E. B. 1952 Action of bacteriophage on mixed strain starter cultures. I. Nature and characteristics of the "nascent phenomenon." J. Dairy Sci., **35**, 371–380.

COLLINS, E. B. 1955 Action of bacteriophage on mixed strain cultures. IV. Domination among strains of lactic streptococci. Appl. Microbiol., **3**, 141–144.

COLLINS, E. B. 1957 A simple method for detecting inhibitory substances in milk. Milk Prods. J., **48**, 48.

COLLINS, E. B. 1958 Manufacturing cottage cheese with pairs of single-strain cultures of lactic streptococci. J. Dairy Sci., **41**, 492–501.

CZULAK, J. AND HAMMOND, L. A. 1954 Compatibility amongst strains of lactic streptococci. Australian J. Dairy Technol., **9**, 15–18.

CZULAK, J. AND NAYLOR, J. 1956 Host-phage relationship of cheese starter organisms. III. Significance in selection and maintenance of starter cultures in commercial use. J. Dairy Research, **23**, 131–133.

HIRSCH, A. 1952 The evolution of the lactic streptococci. J. Dairy Research, **19**, 290–293.

HIRSCH, A. AND GRINSTED, E. 1951 The differentiation of the lactic streptococci and their antibiotics. J. Dairy Research, **18**, 198–204.

HOYLE, M. AND NICHOLS, A. A. 1948 Inhibitory strains of lactic streptococci and their significance in the selection of cultures for starter. J. Dairy Research, **15**, 398–408.

HUNTER, G. J. E. 1946 A simple agar medium for the growth of lactic streptococci. The role of phosphate in the medium. J. Dairy Research, **14**, 283–290.

LIGHTBODY, L. G. AND MEANWELL, L. J. 1955 The growth of lactic acid streptococci in mixed starter cultures. J. Appl. Bacteriol., **18**, 53–65.

MATTICK, A. T. R. AND HIRSCH, A. 1947 Further observations on an inhibitory substance (nisin) from lactic streptococci. Lancet, **153**, 5–8.

NICHOLS, A. A. AND INESON, P. J. 1947 Cheese starter "recovery" after attack by bacteriophage (strain dominance in multiple strain starters). J. Dairy Research, **15**, 99–111.

OXFORD, A. E. 1944 Diplococcin, an anti-bacterial protein elaborated by certain milk streptococci. Biochem. J., **38**, 178–182.

SWARTLING, P. F. 1951 Biochemical and serological properties of some citric acid fermenting streptococci from milk and dairy products. J. Dairy Research, **18**, 256–267.

WHITEHEAD, H. R. 1953 Bacteriophage in cheese manufacture. Bacteriol. Revs., **17**, 109–123.

Microbiological Production of Chlorogenicase

J. C. LEWIS AND P. A. THOMPSON

Western Regional Research Laboratory,[1] *Albany, California*

Received for publication August 10, 1960

Chlorogenicase, an esterase that converts chlorogenic acid to caffeic acid plus quinic acid, was desired for technological research on enzymatic browning of fruit. This paper describes its production by strains of "black yeasts," *Aureobasidium (Pullularia) pullulans* (De Bary) Arnaud (Cooke, 1959), and by strains of *Aspergillus oryzae* and other aspergilli. Special attention was devoted to chemical inducers of the biosynthesis and to solubilization of the enzyme.

MATERIALS AND METHODS

Assay of chlorogenicase. A mixture of 1 ml of 1 per cent chlorogenic acid (0.028 M) in potassium phosphate (0.073 M) at pH 6.0 and 0.5 ml (or less) of enzyme preparation with a few crystals of thymol was incubated at 35 C. The mixture was spotted periodically to Whatman no. 1 filter paper. The paper was developed in *n*-butyl alcohol:acetic acid:water (4:1:1) which gave R_F 0.65 for chlorogenic acid and 0.85 for caffeic acid on inspection under ultraviolet light. Buffered solutions of equimolar amounts of the acids gave roughly similar fluorescence intensities. The chromatograms were scored by visual comparison with standards. First order rate constants were calculated by

$$k^1 = \frac{-\ln (R_2/R_1)}{t_2 - t_1}$$

where R_1 and R_2 are the fractions of original chlorogenic acid unconverted at times t_1 and t_2 (in days). The potency to be attributed to undiluted whole culture, $k_{wc}{}^1$, was obtained by multiplying k^1 by the appropriate concentration factor.

Substitution of phosphate by citrate buffer gave a slow, bright yellowish-fluorescing spot on chromatography. It was shown to represent a dissociable complex of citrate with chlorogenate by cross migration in the same solvent. Toluene inhibited the chlorogenicase of "black yeast" (*A. pullulans*) autolysate markedly. Thymol seemed to be inert, and pH 5.9 to 6.3 appeared much better than pH 5.0 for the "black yeast" autolysate; the use of citrate or phosphate buffer did not affect the rate noticeably.

[1] A laboratory of the Western Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.