CHEMICAL PRESERVATIVES IN FOODSTUFFS

The effect on moulds II.

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The antimicrobial effect of preservatives and the mechanism of their action was discussed extensively in the first paper (5) of this series in which also the relative efficiencies of several preservatives against various groups of microbes were compared. In the present paper are presented the experimental results of a study of the efficiency of various chemical preservatives against a number of moulds that are responsible for the spoilage of foodstuffs.

Methods

The moulds were isolated from spoiled fish preserves, from spices employed in the preparation of the preserves and from various sites of contamination in the cannery. The following culture media proved to be the most suitable for growing the moulds: Czapek's solution + 3 % saccharose + 1.5 % agar-agar (pH 5.4) and Difco bacto malt extract agar (pH 4.6). Czapek's solution contains 2 g $NaNO_3$, 1 g KH_2PO_4, 0.5 g $MgSO_4$. 7 H_2O, 0.5 g KCl and 0.01 g $FeSO_4$. 7 H_2O dissolved in one litre of tap water.

In order to avoid the physiological degeneration of the pure cultures the transfer method in common use in microbiological work was employed (2, p. 191).

For the characterization of the isolated mould species, the growth of colonies on Czapek, malt extract and wort agar in Petri dishes and slant cultures was followed. Particular attention was paid to the colours of the colonies and the colour changes seen in them, the surface structure, the changes in the colours of the media and the appearance of transpiration drops. The morphological properties of the vegetative and fertile hyphae and of the reproductive organs were studied in lactophenol preparations and in Henrici's slide cultures.

In the classification of the moulds Rabenhorst's system (Smith 8) was followed. The following moulds were identified, in some cases only the genus, in other cases also the species being determined:

A_1	*Aspergillus*	sp.	P_1	*Penicillium*	*expansum*
A_2	»	*glaucus*	P_2	»	*chrysogenum*
A_3	»	*clamydosporii*	P_3	»	sp.
A_4	»	sp.	P_4	»	*brevi-compactum*
A_5	»	*niger*	P_5	»	*viridatum*
C_1	*Cladosporium*	*herbanum*	S_1	*Sporotrichum*	sp.

For the study of the relative efficiencies of the preservatives, solid culture media (1.5 % agar) were found more suitable than liquid media. The following method was developed for the purpose.

Three sterilized filter paper circles (Munktell No. 20 S) 1 cm in diameter were placed aseptically on the surface of a buffered (McIlvaine) Czapek or wort agar medium in a Petri dish. One drop of a spore suspension was then added to each paper circle. The dishes were then incubated at 25° C 4, 8 and 12 days. The sizes of the colonies and extent of spore formation were used to evaluate the growth (cf. Brancato and Golding 1).

The preservatives studied were dehydroacetic, sorbic and benzoic acids, ethyl p-hydroxybenzoate, hexamethylenetetramine and 2-methyl-4-amino-1-naphthol hydrochloride (vitamin K_5). The pH values of the culture media were adjusted to 4.55 and 7.

Experimental results

The effect of the preservatives on 12 different mould strains in Czapek's medium and wort agar at pH 4.55 is shown by the data in Table 1. The concentrations of the preservatives required to arrest the mould growth were relatively low at this pH. Dehydroacetic acid was found to be the most effective of the acid preservatives. Of the other preservatives vitamin K_5 was almost as effective. Next in order of effectiveness were: hexamethylenetetramine, sorbic acid, ethyl p-hydroxybenzoate, and benzoic acid. The composition of the culture medium exerted an effect only in the case of benzoic acid and hexamethylenetetramine. The required effective concentrations of these latter preservatives were greater in the wort than in Czapek's medium. For instance, the strains A_4 and P_1 required twice the concentration of benzoic acid in the former medium before their growth was arrested. Also about twice the concentration of hexamethylenetetramine was necessary to prevent the growth of strains A_4, P_2, P_4 and S_1, and about three times the concentration to prevent growth of strains A_2 and A_5 in wort medium than in Czapek's medium. When the tolerances of various strains to the same preservatives were compared, differences were noted in the case of sorbic and benzoic acids and hexamethylenetetramine. *Cladosporium herbanum* (C_1) and *Sporotrichum* sp. (S_1) tolerated 0.008 g of sorbic acid per 100 ml of culture medium, but *Aspergillus niger* (A_5) a ten times

Table 1. The effect of preservatives on the growth of moulds in buffered Czapek's and wort media at pH 4.55. Threshold concentrations of preservatives are given in grams per 100 ml in culture media where no growth was observed. Temperature: 25°C. Buffer: McIlvaine's citric acid-phosphate buffer.

Strain	Time in days	Dehydroacetic acid		Sorbic acid		Benzoic acid		Ethyl ester of p-hydroxy-benzoic acid		Hexamethy-lenetetramine		Vitamin K_5	
		Czapek	wort	Czapek	wort	Czapek	wort	Czapek	wort	Czapek	wort	Czapek	wort
A_1	4	<0.005	<0.005	0.02	0.04	0.06	0.10	0.03	0.04	0.025	0.035	0.005	0.008
	12	0.008	0.008	0.04	0.04	0.08	0.12	0.03	0.04	0.025	0.035	0.008	0.008
A_2	4	<0.005	<0.005	0.01	0.01	0.02	0.03	0.02	0.02	0.005	0.015	0.005	0.005
	12	<0.005	<0.005	0.02	0.02	0.02	0.03	0.03	0.04	0.008	0.025	0.005	0.005
A_3	4	<0.005	<0.005	0.02	0.02	0.04	0.04	0.02	0.02	0.008	0.015	0.005	0.005
	12	<0.005	<0.005	0.02	0.02	0.04	0.06	0.03	0.04	0.015	0.015	0.005	0.005
A_4	4	<0.005	<0.005	0.02	0.02	0.04	0.06	0.03	0.04	0.005	0.015	0.005	0.005
	12	<0.005	<0.005	0.03	0.03	0.05	0.10	0.03	0.04	0.008	0.015	0.005	0.005
A_5	4	<0.005	0.008	0.06	0.06	0.06	0.10	0.02	0.04	0.005	0.015	0.005	0.008
	12	0.008	0.008	0.08	0.08	0.08	0.12	0.04	0.04	0.008	0.025	0.005	0.008
C_1	4	<0.005	<0.005	<0.005	0.008	0.02	0.02	0.02	0.03	0.035	0.050	0.005	0.008
	12	0.005	0.005	0.008	0.008	0.02	0.02	0.03	0.03	0.050	0.080	0.005	0.008
P_1	4	<0.005	0.005	0.02	0.04	0.06	0.10	0.03	0.03	0.025	0.035	0.005	0.008
	12	<0.005	0.008	0.04	0.04	0.06	0.12	0.03	0.03	0.025	0.035	0.005	0.008
P_2	4	<0.005	<0.005	0.02	0.02	0.03	0.04	0.02	0.02	0.008	0.015	0.005	0.008
	12	0.008	0.008	0.02	0.02	0.04	0.06	0.03	0.04	0.015	0.035	0.005	0.008
P_3	4	<0.005	<0.005	0.03	0.03	0.06	0.12	0.02	0.03	0.015	0.015	0.008	0.008
	12	0.008	0.008	0.03	0.04	0.08	0.12	0.03	0.03	0.015	0.015	0.008	0.008
P_4	4	<0.005	<0.005	0.01	0.02	0.03	0.04	0.02	0.02	0.008	0.015	0.005	0.005
	12	<0.005	0.005	0.02	0.02	0.04	0.06	0.03	0.04	0.015	0.035	0.005	0.005
P_5	4	<0.005	<0.005	0.04	0.04	0.05	0.08	0.02	0.04	0.015	0.035	0.005	0.008
	12	<0.005	<0.005	0.04	0.04	0.06	0.10	0.04	0.04	0.025	0.035	0.005	0.008
S_1	4	<0.005	<0.005	0.005	0.005	0.02	0.01	0.01	0.01	0.005	0.015	0.005	0.005
	12	<0.005	<0.005	0.008	0.008	0.02	0.02	0.02	0.02	0.008	0.015	0.005	0.005

greater concentration. The other *Aspergillus* and *Penicillium* strains were about equally as sensitive to sorbic acid. The differences between the strains with respect to benzoic acid and hexamethylenetetramine were generally smaller, but also less uniform than with respect to sorbic acid. *Cladosporium herbanum* (C_1) differed from the other strains in that it was least sensitive to benzoic acid, but was most resistant to hexamethylenetetramine.

In Table 2 the dependence of the efficiencies of the preservatives on the pH is shown. Only the efficiencies of the acid preservatives are seen to vary with the pH. When the pH is 7, considerably larger concentrations of the acid preservatives are required to arrest the growth of moulds than in acid pH. This is due, as has been pointed out previously (5), to the increased dissociation of the acids with increasing

Table 2. The effect of preservatives on the growth of moulds in buffered Czapek's medium at pH 4.55 and 7. Threshold concentrations of preservatives are given in grams per 100 ml in culture media where no growth was observed. Temperature 25°C. Buffer: McIlvaine's citric acid-phosphate buffer.

Strain	Time in days	Dehydroacetic acid		Sorbic acid		Benzoic acid		Ethyl ester of p-hydroxy-benzoic acid		Hexamethylene-tetramine		Vitamin K_5	
		pH		pH		pH		pH		pH		pH	
		4.55	7.00	4.55	7.00	4.55	7.00	4.55	7.00	4.55	7.00	4.55	7.00
A_2	4	<0.005	0.06	0.01	0.4	0.02	0.8	0.02	0.02	0.005	0.01	<0.005	<0.005
	12	<0.005	0.07	0.02	0.7	0.02	1.4	0.03	0.03	0.008	0.02	<0.005	<0.005
A_5	4	<0.005	0.13	0.06	2.0	0.06	3.8	0.02	0.03	0.005	0.01	<0.005	<0.005
	12	0.008	0.15	0.08	2.0	0.08	4.0	0.04	0.03	0.008	0.02	<0.005	<0.005
C_1	4	<0.005	0.08	<0.005	0.1	0.02	1.1	0.02	0.02	0.035	0.24	<0.005	<0.005
	12	0.005	0.15	0.008	0.3	0.02	1.4	0.03	0.02	0.050	0.30	<0.005	<0.005
P_1	4	<0.005	0.15	0.02	0.7	0.06	3.9	0.03	0.03	0.025	0.06	<0.005	<0.005
	12	<0.005	0.15	0.04	0.7	0.06	4.0	0.03	0.03	0.025	0.06	<0.005	<0.005
P_4	4	<0.005	0.08	0.01	0.5	0.03	3.3	0.02	0.02	0.008	0.01	<0.005	<0.005
	12	<0.005	0.15	0.02	0.7	0.04	3.4	0.03	0.03	0.015	0.03	<0.005	<0.005

pH whereupon the microbiostatically active undissociated fraction decreases. The data in Table 2 show also that the efficiency against moulds decreases with increasing acid strength, i.e. with increasing dissociation. Thus at pH 7, 0.15 g dehydroacetic, 2.0 g of sorbic, and 4.0 g of benzoic acid are required per 100 ml of culture medium to arrest the growth of *A. niger* (A_5). Also the efficiency of hexamethylenetetramine is slightly lower at pH 7, but ethyl p-hydroxybenzoate and vitamin K_5 are equally effective in neutral and in acid medium. The order of efficiency at pH 7 is thus: vitamin K_5 > ethyl p-hydroxybenzoate $\simeq$ hexamethylenetetramine > dehydroacetic acid > sorbic acid > benzoic acid. When the relative resistances of the various mould strains to the same preservative were compared, approximately the same differences were noted at pH 7 as at pH 4.55. In addition to sorbic and benzoic acids and hexamethylenetetramine, differences in tolerance are noted with respect to dehydroacetic acid at pH 7. This is quite natural since, owing to the increase in the degree of dissociation, the threshold concentration of dehydroacetic acid is greater in neutral than in acid solution.

In Table 3 are given the threshold concentrations of the acid preservatives and the corresponding undissociated fractions that were required to inhibit the growth of *A. niger* (A_5), *C. herbanum* (C_1) and *P. expansum* (P_1) at pH 4.55 and pH 7. Also the threshold concentrations of ethyl p-hydroxybenzoate and vitamin K_5, whose action is not influenced by pH, have been included in the table. When only the concentrations of the undissociated, microbiostatically active fractions are compared, it is seen that they are lower in neutral than in acid medium, which is in accordance with earlier observations (6). It is also evident that dehydroacetic acid and vitamin K_5 exert a specific action against the moulds in question. The

Table 3. The effect of preservatives on the growth of Aspergillus niger, Cladosporium herbanum and Penicillium expansum in buffered Czapek's medium at pH 4.55 and pH 7.00. Threshold concentrations of preservatives are given in milligrams per 100 ml in culture media where no mould growth was observed.

Preservative	Aspergillus niger (A_8)				Cladosporium herbanum (C_1)				Penicillium expansum (P_1)			
	pH 4.55		pH 7.00		pH 4.55		pH 7.00		pH 4.55		pH 7.00	
	Total concn.	Concn. of undiss. fr.	Total concn.	Concn. of undiss. fr.	Total concn.	Concn. of undiss. fr.	Total concn.	Concn. of undiss. fr	Total concn.	Concn. of undiss. fr.	Total concn.	Concn. of undiss. fr.
Dehydroacetic acid	8	7	150	3	5	4	150	3	5	5	150	3
Sorbic acid	80	50	2000	12	8	5	300	2	40	25	700	4
Benzoic acid	80	24	4000	6	20	5	1400	2	60	20	4000	6
Ethyl ester of p-hydroxybenzoic acid	40		30		30		20		30		30	
Vitamin K_5	5		5		5		5		5		5	

concentration of undissociated benzoic acid required to arrest the growth of *A. niger* and *P. expansum* at pH 4.55 is lower than the corresponding concentrations of undissociated sorbic acid and ethyl p-hydroxybenzoate. The order of specific efficiency at pH 4.55, namely, dehydroacetic acid, vitamin K_5, benzoic acid, sorbic acid and ethyl p-hydroxybenzoate, is hence slightly different from the order seen in Table 1. Owing to the dissociation, the actual total threshold concentration of benzoic acid is, however, even at pH 4.55 greater than the concentrations of the other preservatives studied. The concentration of benzoic acid required to arrest mould growth in neutral medium is already several units per cent. Also the total concentration of dehydroacetic acid required at pH 7 is five times as great as that of ethyl p-hydroxybenzoate, and 30 times as great as that of vitamin K_5. The last-mentioned compound is both absolutely and specifically very effective against moulds.

Discussion

The above experimental results show that relatively low concentrations of preservatives are generally able to arrest the growth of moulds, especially in acid medium. The differences in the efficiencies of the acid preservatives are more pronounced in neutral media, which is due to differences in their degree of dissociation. The most effective and specific inhibitors of mould growth are vitamin K_5 and dehydroacetic acid. Amounts of vitamin K_5 smaller than 0.005 g per 100 ml of culture medium are sufficient to arrest the growth of all mould strains studied at both pH 4.55 and pH 7. The amount of dehydroacetic acid required to effect complete mould growth inhibition at pH 4.55 is 0.005—0.008 g per 100 ml of culture media. As the pH approaches 7 the absolute efficiency of dehydroacetic acid diminishes so that

it is less effective than hexamethylenetetramine and ethyl p-hydroxybenzoate. On the other hand, dehydroacetic acid is so weak (cf. ref. 5, Table 1 and Fig. 1) that its threshold concentration is fairly low, 0.06—0.15 g per 100 ml of culture medium, even at pH 7, while the corresponding concentrations of sorbic and benzoic acids are over ten times as high (Tables 2 and 3). In acid solution also sorbic acid becomes so much more effective that, after vitamin K_5, dehydroacetic acid and hexamethylenetetramine, it is almost as effective as ethyl p-hydroxybenzoate.

The observed threshold concentration values differ in some degree from the values previously reported in the literature. The value for hexamethylenetetramine at pH 7 deviates considerably from the value reported by v. SCHELHORN (7). According to the latter author, 5 per cent hexamethylenetetramine is required to arrest the growth of *Penicillium* sp. in malt peptone meat broth medium; this concentration is appreciably higher than that recorded in the present study. The activity of hexamethylenetetramine is due to the formaldehyde formed by its decomposition, which decomposition is very slow and irregular in neutral medium. Conducted analyses showed that at pH 4.55 all the hexamethylenetetramine added decomposed during the sterilization, but at pH 7 only one–third. When the moulds were grown on a culture media to which the hexamethylenetetramine was added after the sterilization, they were able to tolerate well hexamethylenetetramine concentrations of 4—5 per cent. The moulds are in this case able to utilize this preservative as a source of carbon and nitrogen (3). The observed deviations in the threshold concentrations may hence be due either to the different rates of decomposition of hexamethylenetetramine or to the different culture media.

The composition of the culture medium is known to have an influence on the efficiency of both benzoic acid and hexamethylenetetramine as inhibitors of mould growth. In wort medium which contains organic nitrogen compounds, greater amounts of these preservatives are required to inhibit mould growth than in Czapek's medium (Table 1). Preliminary experiments that we have performed have shown that certain amino acids lower the mould growth inhibiting properties of hexamethylenetetramine. Wort contains in addition factors that are necessary for the growth of microorganisms.

Differences were noted in the resistance of various mould strains to dehydroacetic, sorbic and benzoic acids and hexamethylenetetramine. The differences were particularly marked in the case of sorbic acid, since alongside poorly resistant strains, *Cladosporium herbanum* (C_1) and *Sporotrichum* sp. (S_1), ten times more resistant *Aspergillus niger* strains (A_5) were encountered. This state of affairs may be due to the possibility that the moulds are capable of utilizing the preservatives in their metabolism, since MELNIK and co-workers (4) have found that the disappearance of sorbic acid from packing materials used for cheeses is due to the oxidative metabolism of moulds.

REFERENCES:

(1) Brancato, F. P. & Golding, N. S. 1953. The diameter of the mold colony as a reliable measure of growth. Mycologia (N.Y.) 14: 848—891. Ref. 1955. Z. Lebensm. Unters. u. Forsch. 100: 421.
(2) Foster, J. W. 1949. Chemical activities of fungi. New York.
(3) Giesecke & Tseretheli, O. von. 1940. The utilization of hexamethylenetetramine by soil microorganisms. Bodenkunde u. Pflanzenernähr. 21/22: 408, Ref. 1943. Chem. Abstr. 37: 6300.
(4) Melnick, D. & Luckmann, F. H. & Gooding, C. M. 1954. Sorbic acid as a fungistatic agent for foods. VI. Metabolic degradation of sorbic acid in cheese by molds and the mechanism of mold inhibition. Food Res. 19: 44—58.
(5) Nikkilä, O. E. & Linko, R. R. 1958. Elintarvikkeiden kemiallisista säilöntäaineista I. Antimikrobinen tehokkuus ja vaikutustapa. Reports of the State Institute for Technical Research, Finland.
(6) Schelhorn, M. von. 1953. Efficacy and specificity of chemical food preservatives. Food Technol. 7: 97—101.
(7) Schelhorn, M. von. 1954. Untersuchungen über Konservierungsmittel IX. Hexamethylentetramin als Konservierungsmittel. Deut. Lebensm. Rundschau 50: 90—92.
(8) Smith, G. 1947. An introduction to industrial mycology. London.

SELOSTUS:

ELINTARVIKKEIDEN KEMIALLISISTA SÄILÖNTÄAINEISTA

II. Vaikutus homeisiin

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Pilaantuneista kalasäilykkeistä ja niiden tartuntakohteista säilyketehtaalla eristettiin 12 homekantaa, jotka kuuluivat *Aspergillus-*, *Cladosporium-*, *Penicillium-* ja *Sporotrichum* sukuihin. Näitä koeobjekteina käyttäen vertailtiin eri säilöntäaineiden tehoa Czapekin mediumissa ja vierteessä pH 4.55:ssä ja pH 7:ssä tarkoitukseen kehitetyllä paperikiekkomenetelmällä.

Säilöntäaineiden tehokkuusjärjestys oli pH 4.55:ssä dehydroetikkahappo > K_8-vitamiini > heksametylentetramiini > sorbiinihappo ≂ p-oksibentsoehapon etylesteri > bentsoehappo ja pH 7:ssä K_8-vitamiini > p-oksibentsoehapon etylesteri ≂ heksametylentetramiini > dehydroetikkahappo > sorbiinihappo > bentsoehappo. Vain happamien säilöntäaineiden sekä heksametylentetramiinin teho oli riippuvainen mediumin pH:sta. Homeiden kasvun estämiseen tarvittavat säilöntäainemäärät olivat yleensä melko pieniä.

Ravintoalustan koostumuksella oli vaikutusta sekä bentsoehapon että heksametylentetramiinin tehokkuuteen. Vierteessä tarvittiin näitä säilöntäaineita enemmän kuin synteettisessä Czapekin mediumissa. Tämä johtuu vierteen sisältämistä N-yhdisteistä ym. kasvutekijöistä. Niinpä todettiin, että määrätyillä aminohapoilla oli antagonistinen vaikutus heksametylentetramiiniin.

Eri homekantojen resistenttisyydessä havaittiin eroja dehydroetikka-, sorbiini- ja bentsoehapon sekä heksametylentetramiinin suhteen. Erikoisen selvänä tämä ilmeni sorbiinihapon suhteen.

Tutkituista säilöntäaineista oli K_8-vitamiini sekä absoluuttiselta että spesifiseltä vaikutukseltaan tehokkain. Voimakkaita homemyrkkyjä olivat myöskin dehydroetikkahappo sekä heksametylentetramiini, vaikkakin viimeksimainittua on tässä suhteessa yleisesti pidetty melko tehottomana.
