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PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 7 (12): 2198-2201, 2004
ISSN 1028-8880

Inhibitory Activity of Micro-organisms Isolated from Wine Bottle Corks

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Abstract: Competition between micro-organisms and the production of inhibitor substances by some of these micro-organisms is frequent in substrata with varied, complex microbiota, such as cork. The main aim of this study was to determine the inhibitor activity of filamentous fungi, yeasts and bacteria isolated from wine bottle corks. The inhibitor activity of isolated micro-organisms was determined by placing disks of pure culture in Petri dishes containing 2% malt extract agar for fungi and trypticase soy agar for bacteria. These were surface-seeded beforehand with the test micro-organism, the dishes are incubated in conditions suitable for the growth of the surface-seeded micro-organisms and finally the inhibitor plaques produced are measured. The results indicate that the micro-organisms with the greatest inhibitor activity are *Penicillium citrinum* and *Trichoderma viride.*

Key words: Cork, micro-organisms, inhibitory activity

INTRODUCTION

The particular nature of cork allows the colonisation and maintenance of micro-organisms in cell lenticels or pores, due to which cork has a natural microbiota, which may develop during the wine cork manufacturing process[1-3]

Some species of micro-organisms belonging to the cork microbiota, especially filamentous fungi, are capable of attacking the cork directly or producing metabolites that may affect the quality of the wine corks, the wine that comes into contact with the corks and the health of consumers.

Furthermore, competition between the micro-organisms and the production of inhibitory substances by some micro-organisms is frequent in substrata with a varied, complex microbiota[4], such as cork and, therefore, wine bottle corks.

The presence or absence of certain micro-organisms in this type of substratum depends on a number of factors, in particular: moisture, growth speed and synergetic or antagonistic relationships that may arise among them[5]. Furthermore, the production of metabolites such as enzymes, antibiotics and toxins may influence the capacity of some micro-organisms to inhibit the growth of others[6].

The main aim of this study was to determine the inhibitor activity of filamentous fungi, yeasts and bacteria isolated from wine bottle corks.

MATERIALS AND METHODS

Pure cultures of the species of filamentous fungi, yeasts and bacteria isolated beforehand from wine bottle corks were produced (Table 1) on dishes of 2% Malt Extract Agar with added antibiotic (MEA) for 3-5 days at 28°C. Next, bacterial suspension cultures with an optical density equal to tube no. 1 on the McFarland scale were prepared on dishes of Trypticase Soy Agar (TSA) for 24 h at 37°C.

After this time, 6 mm diameter disks of the cultures were cut out and placed on Petri dishes containing MEA and TSA, in which cell suspensions had been surface-seeded beforehand using a Digraslky loop, at a concentration of 10^6 cells/mL for all the micro-organisms.

Table 1: Filamentous fungi, yeasts and bacteria isolated from samples of wine bottle corks

Filamentous fungi	Yeasts	Bacteria
Alternaria alternata	*Rhodotorula glutinus*	*Bacillus cereus*
Penicillium citrinum	*Candida ciferri*	*B. circulans*
P. velutinum		*B. firmus*
Aspergillus niger		*B. lentus*
A. fumigatus		*B. pantothenticus*
Mucor plumbeus		*Nocardia* sp.
Trichoderma solani		*Agrobacterium* sp.
Fusarium solani		*Micrococcus* sp.
F. moniliforme		*M. luteus*
Monilia sitophila		*Achromobacter* sp.
		Aeromonas sp.
		Erwina herbicola
		Acinetobacter-lwoffii

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Two disks of each organism were placed in the dishes, one in contact with the microbe development area and the other in contact with the area of culture medium, in order to study the inhibitor activity of the micro-organism. Disks containing only the culture medium were also placed in each dish as controls. In addition, control dishes containing only suspensions of the micro-organism were seeded in order to test viability.

The dishes were incubated in conditions suitable for the growth of surface-seeded micro-organism, i.e. 28°C for 3-5 days in the case of filamentous fungi and yeasts and 37°C for 24 h for bacteria. After this time, the inhibitor plaques produced were measured in the following way: the total inhibitor plaque diameters were measured, the disk diameters were subtracted from this total and the result was divided by two. The results were expressed in millimetres (mm).

RESULTS AND DISCUSSION

The results showed that the filamentous fungi isolated from samples which have the greatest activity against other filamentous fungi and yeasts present in wine bottle corks are *Penicillium citrinum* and *Trichoderma viride* (Table 2). The latter is particularly notable because of its additional capacity to inhibit the sporulation of *Aspergillus niger* and *Penicillium velutinum* and because of its production of larger inhibitor plaques when contact with other micro-organisms is through its mycelial zone.

Trichoderma viride is a filamentous fungus which is characterised by extremely rapid growth (colonies of 4.5 to 7.5 cm in 5 days) and in particular, by its antagonistic and parasitic capacity towards other filamentous fungi, yeasts and bacteria, in particular towards *Fusarium solani* and species of *Mucor* and *Rhizopus*[5-9] as shown in the results of this study. The inhibitor capacity of *Trichoderma viride* arises from the production of antibiotic and antifungal compounds, such as trichodermin, trichothecene and trichotoxin A[9].

The filamentous fungus that proved most sensitive to the inhibitor activity of other filamentous fungi was *Penicillium velutinum*, as not only were inhibitor plaques observed, but also its sporulation capacity was partially or completely affected in most cases. By contrast, *Trichoderma viride* was the most resistant fungus to the inhibitor activity of other fungi.

The filamentous fungi and yeasts that presented the greatest inhibitor activity against bacteria were *Penicillium citrinum* and *Mucor plumbeus*, followed by *Alternaria alternata, Fusarium solani* and *Rhodotorula glutinis* (Table 3). However, all fungi except *Candida ciferri* presented activity against isolated bacteria.

The species of the genus *Bacillus*, in particular *B. lentus* and *B. firmus*, were the most sensitive to filamentous fungi inhibitor activity. By contrast, *Streptomyces* sp. and *Aeromonas* sp. were the least resistant.

Table 2: Inhibitor activity of filamentous fungi and yeasts isolated from wine bottle corks

	Inhibitor zones (mm) culture medium Inhibitor zones (mm) mycelial zone										
Micro-organism	1	2	3	4	5	6	7	8	9	10	11
1	.	-	3.0	-	-	-	4.0	-	-	-	-
		3.0	-	3.0	-	-	5.0	-	-	-	-
2	-	.	3.0	4.0	7.0	6.0	6.0	3.0	3.5	4.0	-
	-		3.5	-	4.0	4.5	5.5	3.0	4.0	7.0	3.0
3	-	-	.	-	-	-	-	-	4.0	-	-
	-	-		-	-	-	-	-	5.5	-	-
4	6.5	-	3.0	.	4.0	-	-	-	5.5	-	-
	4.0	-	3.5		-	-	-	-	-	-	3.5
5	-	5.5	4.0	-	.	-	-	-	4.5	-	5.5
	-	4.5	6.5	-		-	-	-	4.0	-	-
6	-	15.0	6.5	-	-	.	-	-	4.0	-	5.0
	-	7.0	6.5	-	-		-	-	4.0	-	6.0
7	-	9.5	-	-	9.5	5.5	.	8.0	-	-	-
	-	13.5	5.5	9.5	8.5	8.5		8.0	5.0	12.0	17.0
8	-	6.0	4.0	2.5	8.0	1.5	-	.	-	-	-
	-	8.0	5.0	2.5	6.5	4.0	-		-	-	-
9	-	3.0	3.0	-	6.5	2.0	-	-	.	-	5.0
	-	4.0	-	6.5	6.5	2.0	-	-		-	5.0
10	-	-	-	-	5.0	-	-	-	-	.	-
	-	-	-	-	7.0	-	-	-	-		-
11	-	-	-	-	-	-	-	-	2.5	-	.
	-	-	-	-	-	-	-	-	-	-	

1: *Alternaria alternata*, 2: *Penicillium citrinum*, 3: *Penicillium velutinum*, 4: *Aspergillus niger*, 5: *Aspergillus fumigatus*, 6: *Mucor plumbeus*
7: *Trichoderma viride*, 8: *Fusarium solani*, 9: *Fusarium moniliformer*,10: *Monilia sitophila*, 11: *Rhodotorula glutinis*

Table 3: Inhibitor activity of filamentous fungi and yeasts against bacteria

Micro-organism	Inhibitor zones (mm) culture medium / Inhibitor zones (mm) mycelial zone													
	12	13	14	15	16	17	18	19	20	21	22	23	24	25
1	9.5	-	6.0	8.0	4.5	4.5	2.5	-	6.0	-	4.0	5.0	1.5	-
	8.5	-	6.0	6.0	5.0	4.5	2.5	-	7.0	-	4.0	4.0	2.5	-
2	8.0	-	6.0	4.0	4.0	1.5	2.5	7.0	5.0	2.5	-	5.5	2.5	-
	6.0	-	4.5	4.0	7.0	-	2.5	5.0	4.0	-	-	4.0	1.5	-
3	-	-	5.0	6.5	-	1.5	3.0	4.0	-	4.5	2.0	4.5	2.5	-
	-	-	-	-	-	-	-	3.0	-	-	-	2.0	-	-
4	-	-	7.0	5.0	5.0	3.5	2.5	3.0	4.5	-	2.5	-	-	-
	-	-	4.0	4.0	4.5	1.5	-	4.0	3.5	-	3.0	-	3.5	-
5	5.0	-	5.	4.0	5.5	3.5	2.5	5.0	6.0	2.5	-	-	-	-
	9.0	-	4.0	4.0	5.0	1.5	-	4.5	5.5	3.5	2.5	-	-	-
6	9.0	6.0	5.5	4.0	-	3.0	-	5.	4.5	2.5	3.0	3.5	-	6.0
	6.5	6.0	5.0	4.0	1.0	3.0	-	5.0	4.0	1.0	2.5	4.0	-	6.5
7	-	4.5	5.0	6.0	-	-	-	3.5	5.0	-	3.0	-	2.5	-
	-	5.5	4.5	5.5	-	-	-	5.0	6.0	-	-	3.0	5.0	-
8	4.5	3.5	4.0	3.5	-	1.5	3.0	3.5	4.5	3.5	-	3.5	-	-
	3.5	3.0	3.5	3.0	-	-	2.5	4.0	4.0	2.0	-	3.5	-	-
9	-	-	3.5	3.5	5.5	2.0	-	6.5	-	-	1.5	-	-	-
	-	-	3.0	4.5	4.5	1.5	-	7.5	-	-	2.5	-	-	-
10	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-	-	-	-	-	-
11	-	-	4.0	4.5	4.5	1.5	3.0	3.5	-	3.0	-	3.5	2.5	-
	-	-	4.0	4.5	4.5	1.5	3.0	3.5	-	3.5	-	4.0	3.5	-

1: *Alternaria alternata*, 2: *Penicillium citrinum*, 3: *Penicillium velutinum*, 4: *Aspergillus niger*, 5: *Aspergillus fumigatus*, 6: *Mucor plumbeus*
7: *Trichoderma viride*, 8: *Fusarium solani*, 9: *Fusarium moniliformer*, 10: *Monilia sitophila*, 11: *Rhodotorula glutinis*, 12: *Bacillus cereus*
13: *B. circulans*, 14: *B. firmus*, 15: *B. lentus*, 16: *B. pantothenticus*, 17: *Nocardia* sp., 18: *Agrobacterium* sp., 19: *Micrococcus* sp., 20: *M. lutens*
21: *Achromobacter* sp., 22: *Aeromonas* sp., 23: *Erwinia herbicola*, 24: *Acinetobacter lwoffii*, 25: *Streptomyces* sp.

Table 4: Inhibitor activity of bacteria isolated from wine bottle corks

Micro-organism	Inhibitor zones (mm) culture medium / Inhibitor zones (mm) bacterial growth										
	12	14	15	16	17	19	20	21	22	23	24
13	-	-	-	-	5.0	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-	-	-
15	-	4.5	-	-	-	4.0	3.0	-	3.0	-	-
	-	4.5	-	-	-	3.5	3.0	-	1.5	-	-
17	3.5	3.0	2.5	-	-	5.0	6.0	-	2.5	-	-
	3.0	2.5	3.0	-	-	5.0	6.0	-	2.5	-	-
18	-	8.5	-	-	-	11.5	-	3.0	-	-	2.0
	-	4.5	-	-	-	7.5	-	3.0	-	-	1.5
19	-	-	-	-	-	-	-	3.0	-	-	-
	-	-	-	-	-	-	-	3.0	-	-	-
21	-	-	-	5.0	-	-	-	-	-	-	-
	-	-	-	4.0	-	-	-	-	-	-	-
23	-	-	-	-	-	-	-	-	-	-	-
	-	-	3.5	-	-	-	-	-	-	-	-
24	-	5.0	-	-	-	-	-	-	-	6.0	-
	-	2.0	-	-	-	-	-	5.5	-	2.0	-

12: *Bacillus cereus*, 13: *B. circulans*, 14: *B. firmus*, 15: *B. lentus*, 16: *B. pantothenticus*, 17: *Nocardia* sp., 18: *Agrobacterium* sp., 19: *Micrococcus* sp.
20: *M. lutens*, 21: *Achromobacter* sp., 22: *Aeromonas* sp., 23: *Erwinia herbicola*, 24: *Acinetobacter lwoffii*

The low inhibitor activity of isolated bacteria against filamentous fungi and yeasts was observed generally, in comparison with the results obtained from the inhibitor activity of the latter two against bacteria.

Nocardia sp. and *Agrobacterium* sp. presented the greatest activity against other bacteria (Table 4). By contrast, *Bacillus cereus, B. firmus* and *Micrococcus luteus* presented no activity against filamentous fungi, yeasts, or bacteria (Table 4 and 5).

Observations showed that *Agrobacterium* sp. was the bacteria with the greatest inhibitor activity against filamentous fungi and yeasts, characterised by the partial inhibition of the sporulation of some filamentous fungi in contact with the bacteria; the sporulation capacity of *A. niger* and *T. viride* were particularly affected.

Of particular note is the low inhibitor activity of *Monilia sitophilia* (against *A. niger* and *A. fumigatus*) and its resistance to other micro-organisms. The results

Table 5: Inhibitor activity of bacteria against filamentous fungi and yeasts isolated from wine bottle corks

Micro-organism	Inhibitor zones (mm) culture medium / Inhibitor zones (mm) bacterial growth							
	2	3	4	6	7	8	10	21
16	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	2.0	-
17	-	-	-	3.0	-	2.0	-	-
	-	-	-	2.5	-	4.0	-	-
		PIE						
18	-	-	-	-	7.0	-	-	-
	4.0	-	-	-	9.5	-	-	2.5
			PIE					
19	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-
			PIE					
21	-	-	4.0	2.0	-	-	-	-
	-	-	4.0	3.0	-	-	-	-
	PIE							
22	-	-	4.5	-	-	2.0	-	-
	-	-	2.5	-	-	2.5	-	-
			PIE			PIE		
23	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	2.0	-
24	-	-	-	4.0	-	-	-	-
	-	-	-	4.0	-	-	2.0	-
					PIE			
25	-	-	-	3.0	-	-	2.0	-
	-	-	-	2.5	-	-	3.0	-
					PIE			

2: *Penicillium citrinum*, 3: *P. velutinum*, 4: *Aspergillus nige*, 6: *Mucor plumbeus*, 7: *Trichoderma viride*, 8: *Fusarium solani*, 10: *Monilia sitophila* 11: *Rhodotorula glutinis*, 16: *B. pantothenticus*, 17: *Nocardia* sp., 18: *Agrobacterium* sp., 19: *Micrococcus* sp., 21: *Achromobacter* sp., 22: *Aeromonas* sp., 23: *Erwinia herbicola*, 24: *Acinetobacter lwoffii*, 25: *Streptomyces* sp., PIE, Partially Inhibits Sporulation

obtained show that this micro-organism does not produce inhibitor plaques in most micro-organisms, nor is its growth affected by micro-organisms. This suggests that there is competition for space, as shown by its invasive growth.

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