

Screening of Fibrinolytic Enzymes of Microorganisms

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Screening of fibrinolytic enzymes from bacteria, yeasts, molds and basidiomycetes was done. All bacteria except *Serratia plymuthica* IFO 3055 had fibrinolytic activity under aerobic or anaerobic culture conditions. Yeasts cultured under both aerobic and anaerobic conditions dissolved fibrin. All molds cultured under anaerobic conditions dissolved fibrin. On the other hand, fibrinolytic activity of *Pleurotus sp.* was higher in basidiomycetes.

Introduction

Thrombosis is one of the major causes of death in Japan and the western world. Typical thrombolytic agents clinically used therapeutically include urokinase and a tissue-type plasminogen activator (t-PA). On the other hand, recently, Sumi *et al.* have reported that traditional fermented foods, such as natto^{1,2)} and shiokara³⁾, have high fibrinolytic activity. These fermented foods are produced by various microorganisms, which have enzymes such as protease, amylase, alcohol dehydrogenase and lactate dehydrogenase.

Thus, we did a screening of fibrinolytic enzymes of microorganisms.

Materials and Methods

Microorganisms

Sixty-eight bacteria, 34 yeasts, 11 molds and 19 basidiomycetes were used in this study.

Medium and cultivation condition

Bacteria, yeasts and molds were cultured in 300ml Erlenmyer flasks with 100ml of each medium under aerobic (on a rotary shaker, 100rpm) and anaerobic conditions. Basidiomycetes were cultured under aerobic conditions. The culture medium for bacteria consisted of 0.5%

meat extract, 1.0% peptone and 0.5% NaCl (pH 7.0). The culture medium for lactic acid bacteria consisted of 0.24% meat extract, 0.5% yeast extract, 0.05% soluble starch, 1.0% protease peptone, 0.4% Na₂HPO₄, 0.05% glucose and 0.44% liver extract (pH 7.2). The culture medium for yeasts, molds and basidiomycetes consisted of 2% malt extract (pH 5.6). Bacteria were cultured at 28°C for 24h. Lactic acid bacteria were cultured at 37°C for 24h. Yeasts, molds and basidiomycetes were cultured at 28°C for 24h, 2 weeks and 2 weeks, respectively.

After cultivation, the culture broths of bacteria, lactic acid bacteria and yeast were centrifuged (8,000rpm, 20min, 4°C) to collect cells. The culture broths of molds and basidiomycetes were filtered with mesh and collected cells. The cell pellets were washed twice with saline solution, and stored at -20°C until they were used for experiments.

Preparation of cell-free extract

The cell pellet was suspended in 1M potassium phosphate buffer (pH 7.0) and disrupted by sonication below 0~8°C (4 times in 15sec, in the case of basidiomycetes 8~12 times in 15sec). The undestroyed cells and debris were removed by centrifugation at 15,000rpm for 10min at

4°C. The supernatant solutions obtained were used as the cell-free extract.

Assay of fibrinolytic activity

Following the method of Astrup and Müllertz⁴⁾, 4ml of 0.6% bovine fibrinogen (Nacalai tesque) in 0.18M borate buffer (pH 7.75), 2ml of 10U/ml bovine thrombin (mochida pharmaceutical co., LTD) and 0.1% CaCl₂ in borate buffer were mixed in a petri dish. Thirty-microliters of cell-free extract were placed on each fibrin plate and the plates were incubated at 37°C for 18h. Fibrinolytic activity was represented by areas of clear zone (major axis ×

minor axis [mm²]).

Measurement of protein concentration

Protein concentration was measured using Bio-Rad Protein Assay.

Results and Discussion

Figure 1 shows the effects of cell-free extract from various microorganisms on fibrinolytic activity. The effects of cell-free extract from bacteria, yeasts, molds and basidiomycetes on fibrinolytic activity are summarized in Table 1, 2, 3 and 4, respectively.

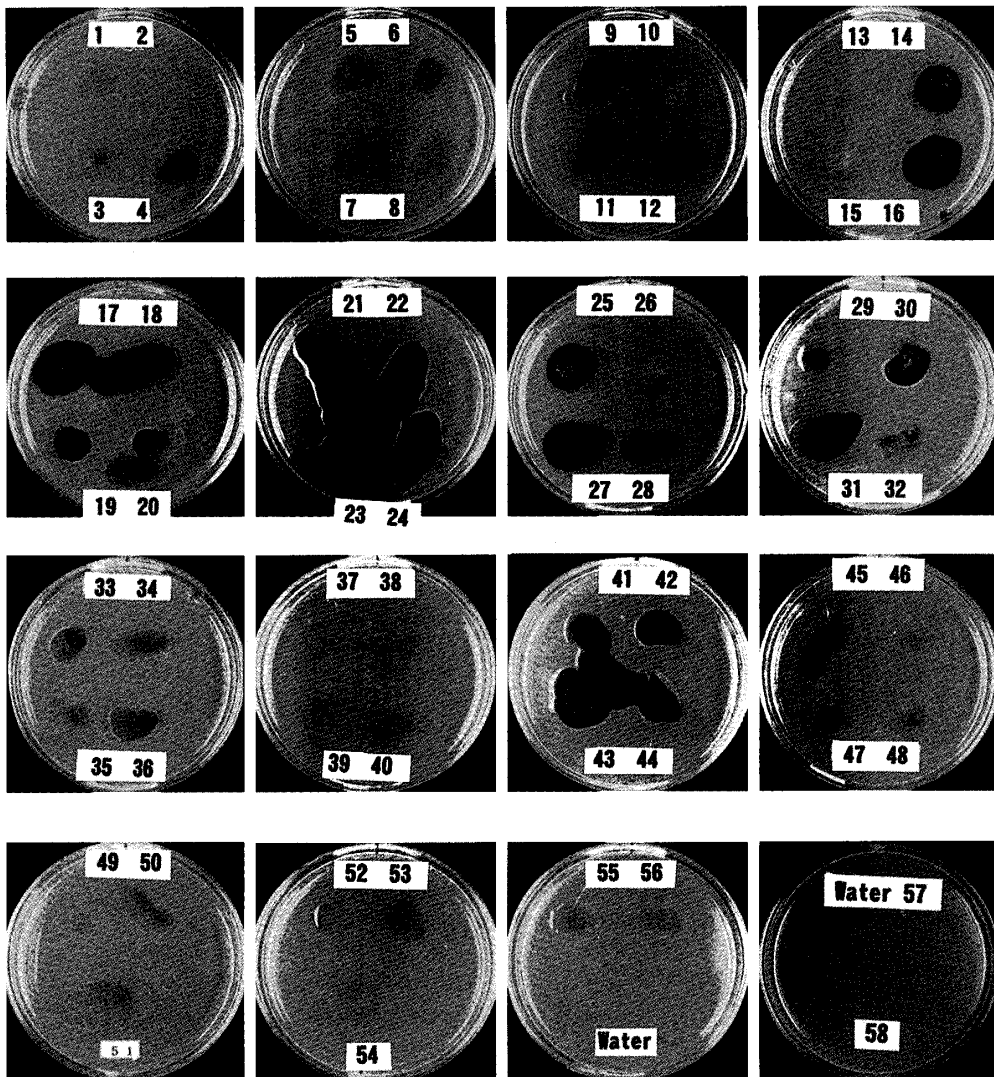


Fig. 1. Fibrinolytic activity of microorganisms.

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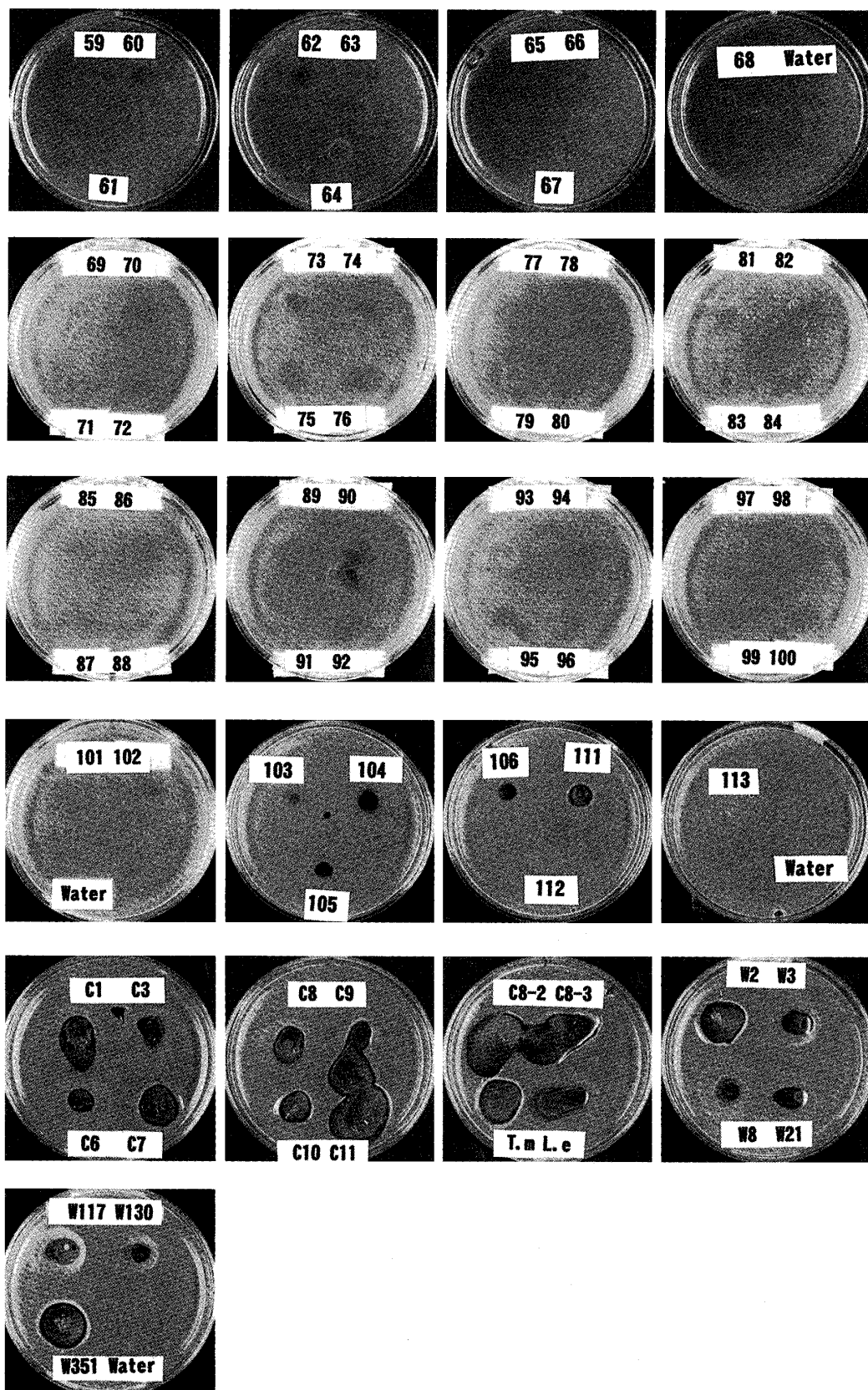


Fig. 1. Fibrinolytic activity of microorganisms.

Photographs show dissolved zone of crude enzymes on fibrinplates.

Table 1. Fibrinolytic activity of bacteria

Strain No.	Bacteria	Fibrinolytic activity mm ²		Protein (mg/ml)		Condition*	
		Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic
1	<i>Agrobacterium radiobacter</i> IAM 1526	212.04	643.47	1.91	1.96	DW	C
2	<i>Agrobacterium tumefaciens</i> IAM B-26-1	0.00	540.00	2.00	1.99	—	CD
3	<i>Alcaligenes faecalis</i> IAM B-141-1	165.39	881.60	2.03	2.02	DW	C
4	<i>Alcaligenes polymorpha</i>	303.00	409.86	1.83	1.99	D	C
5	<i>Arthrobacter globiformus</i> IFO 12137	307.84	459.68	1.79	2.00	D	W
6	<i>Arthrobacter oxydans</i> IFO 12138	231.40	510.51	1.81	1.91	CD	C
7	<i>Arthrobacter pascens</i> IFO 12139	214.24	574.86	1.98	1.92	D	CD
8	<i>Arthrobacter simplex</i> IFO 3530	116.83	617.05	1.92	1.81	D	W
9	<i>Arthrobacter ureafaciens</i> IFO 12140	455.28	628.00	1.77	2.00	W	C
10	<i>Bacillus brevis</i> IFO 3331	105.00	248.00	2.05	1.98	W	W
11	<i>Bacillus cereus</i> IFO 3001	143.78	566.10	1.96	1.72	CD	W
12	<i>Bacillus megaterrium</i> NI 8100 NTH B12	191.90	204.49	2.00	2.01	CD	CD
13	<i>Bacillus pumilus</i> IFO 3030	0.00	331.66	2.04	1.90	—	C
14	<i>Bacillus sphaericus</i> IFO 3525	353.28	334.40	2.04	2.00	C	C
15	<i>Bacillus brevis</i> IFO 3007	99.90	351.52	2.02	2.00	W	C
16	<i>Bacterium cadaveris</i> IFO3731	510.00	118.80	2.02	2.01	C	C
17	<i>Bacterium ketoglutamicus</i> SO(1)Shionogi Co., Ltd	552.82	0.00	1.85	2.02	C	—
18	<i>Bacterium orleanense</i> IFO 3259	545.37	336.00	1.96	1.97	C	W
19	<i>Brevibacterium sp.</i> P145 Phage host	229.60	0.00	1.89	2.01	C	—
20	<i>Brevibacterium sp.</i> P145 N.Kato	760.50	240.80	1.65	1.92	DW	C
21	<i>Corynebacterium fascians</i> IAM 1079	954.80	360.36	1.91	1.99	W	CD
22	<i>Corynebacterium glutamicum</i> No.534 ATCC 13032	642.20	421.80	2.00	2.01	C	W
23	<i>Corynebacterium pseudodiphtheritium</i>	508.95	300.98	1.61	2.01	C	C
24	<i>Enterobacter aerogenes</i> IFO 3320	577.50	191.25	1.95	1.93	C	D
25	<i>Enterobacter cloacae</i> IAM 1221	396.39	608.76	2.03	1.95	C	CD
26	<i>Erwinia arvideae</i> IFO 3830	116.40	789.57	2.02	2.00	W	W
27	<i>Erwinia carotovora</i> IFO 3380	389.15	0.00	2.02	2.02	C	—
28	<i>Escherichia coli</i> K12 IFO 3208	480.48	543.15	1.87	1.95	D	C
29	<i>Escherichia freundii</i> S-96	253.30	348.21	1.93	1.93	D	CD
30	<i>Escherichia intermedia</i> A-21	298.20	370.80	1.59	1.98	C	C
31	<i>Klebsiella pneumoniae</i> IFO 3317	453.18	0.00	1.98	1.96	C	—
32	<i>Klebsiella pneumoniae</i> IFO 12009	454.14	312.00	1.70	1.96	DW	W
33	<i>Klebsiella pneumoniae</i> IFO 12019	303.36	722.70	1.91	1.92	D	C
34	<i>Klebsiella pneumoniae</i> IFO 12932	483.45	267.80	1.84	1.87	D	D
35	<i>Klebsiella pneumoniae</i> IFO 13541	66.50	520.30	1.95	1.96	D	D
36	<i>Klebsiella pneumoniae</i> IFO 13703	252.98	238.29	1.92	1.98	D	CD
37	<i>Micrococcus luteus</i> IFO 3763	0.00	964.48	1.84	1.47	D	C
38	<i>Micrococcus lysodeikticus</i> Fleming IFO 3333	0.00	715.50	1.95	1.88	D	C
39	<i>Micrococcus roseus</i> IFO 3764	0.00	279.84	1.82	1.95	D	C
40	<i>Micrococcus rubens</i> IFO 3768	0.00	426.30	1.79	1.96	D	C
41	<i>Proteus mirabilis</i> IFO 3849	291.90	401.36	1.84	2.01	C	D
42	<i>Proteus morgani</i> IFO 3168	286.50	505.80	1.93	2.02	CD	CD
43	<i>Proteus vulgaris</i> IFO 3988	1045.60	165.39	2.00	1.95	C	D
44	<i>Pseudomonas fluorescens</i> IFO 3081	289.94	550.08	1.90	1.96	C	C
45	<i>Pseudomonas fragi</i> IFO 3458	341.89	299.72	1.78	2.02	CD	D
46	<i>Pseudomonas graveolens</i> IFO 3460	0.00	359.55	1.88	1.97	—	D
47	<i>Pseudomonas iodium</i> IFO 3558	81.90	330.75	1.78	2.02	C	C
48	<i>Pseudomonas solanacearum</i> IFO 3509	34.80	0.00	1.94	2.04	W	—
49	<i>Pseudomonas striafaciens</i> IFO 3309	0.00	350.00	2.03	1.92	DW	W
50	<i>Sarcina aurantiaca</i> IFO 3064	562.40	214.60	1.82	2.04	DW	W
51	<i>Sarcina lutea</i> IFO 1099	0.00	461.27	1.87	2.03	DW	DW
52	<i>Serratia liquefaciens</i> IFO 12979	114.95	915.42	2.02	2.02	W	C
53	<i>Serratia marcescens</i> IFO 3054	223.20	443.45	1.90	1.81	D	C
54	<i>Serratia plymuthica</i> IFO 3055	0.00	0.00	1.96	1.43	—	—
55	<i>Staphylococcus aureus</i> IFO 3060	298.98	0.00	1.86	1.28	W	—
56	<i>Staphylococcus epidermidis</i> IFO 3762	244.42	336.00	1.98	1.95	D	DW
57	<i>Bifidobacterium adolescentis</i> M101-4	271.25	379.08	1.56	2.04	DW	C
58	<i>Bifidobacterium bifidum</i> A234-4	323.99	226.56	1.61	2.04	DW	W
59	<i>Bifidobacterium breve</i> I-53-8	282.00	281.40	1.63	1.87	DW	W
60	<i>Bifidobacterium infantis</i> I-10-5	342.00	540.69	1.47	1.88	DW	C
61	<i>Bifidobacterium longum</i> M101-2	366.00	387.36	1.36	1.99	D	W
62	<i>Lactobacillus bulgaricus</i> IFO 13953	299.15	604.15	1.82	1.93	DW	CD
63	<i>Lactobacillus clerbueckii</i> IFO 3202	268.20	422.44	1.32	1.66	DW	DW
64	<i>Lactobacillus paracasei</i> IFO 3953	300.51	534.63	1.65	2.01	DW	C
65	<i>Lactococcus cremoris</i> IFO 3427	272.32	588.28	1.45	1.91	DW	DW
66	<i>Lactococcus lactis</i> IFO 12007	324.00	381.33	1.46	1.85	DW	CD
67	<i>Pediococcus acidilactici</i> IFO 3888	—	363.80	—	1.91	—	CD
68	<i>Streptococcus thermophilus</i> IFO 13957	507.02	394.94	1.51	1.81	D	DW

*. The clear zone was decided as follows : C, clear ; D, a little muddy ; W, muddy white ; CD, middle clear zone between C and D ; DW, middle clear zone between D and W

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Table 2. Fibrinolytic activity of yeasts

Strain No.	Yeasts	Fibrinolytic activity mm ²		Protein (mg/ml)		Condition*	
		Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic
69	Awamori yeast (Sakamoto)	31.32	126.00	1.51	0.20	CD	W
70	<i>Candida guilliermondii</i> IFO 0566	19.35	120.00	1.72	0.22	W	CD
71	<i>Candida pelliculosa</i> IFO 0707	15.00	55.25	1.67	0.19	D	D
72	<i>Candida utilis</i> IFO 0619	81.00	168.00	1.77	0.13	W	C
73	<i>Cryptococcus albidus</i> IFO 0378	507.30	75.21	1.36	0.06	C	C
74	<i>Cryptococcus laurentii</i> IFO 0609	100.00	110.60	1.55	0.32	W	C
75	<i>Cryptococcus neoformans</i> IFO 0410	68.20	210.00	1.16	0.14	C	C
76	<i>Debaryomyces japonicus</i> IFO 0039	83.52	133.00	1.61	0.32	W	C
77	<i>Debaryomyces vini</i> Y.U.	123.50	119.00	1.50	0.06	DW	D
78	<i>Endomycopsis capsularis</i> IFO 0672	71.39	72.00	1.60	0.07	W	D
79	<i>Endomyces decipiens</i> IFO 0102	65.40	72.25	1.56	0.11	D	D
80	<i>Hansenula miso</i> IFO 0146	69.00	64.50	1.40	0.25	W	DW
81	<i>Hansenula suaveolens</i> IFO 0992	70.97	128.96	1.52	0.13	DW	D
82	<i>Hansenula wingei</i> IFO 0976	123.60	135.00	1.59	0.11	DW	CD
83	<i>Kloeckera apiculata</i> IFO 0865	100.10	68.40	1.45	0.10	DW	D
84	<i>Nadsonia fulvescens</i> IFO 0666	45.12	87.50	1.42	0.14	D	D
85	<i>Pichia anomala</i> IFO 0568	89.25	71.20	1.36	0.11	D	DW
86	<i>Pichia orientalis</i> IFO 1279	43.86	71.25	1.50	0.19	D	DW
87	<i>Pichia polymorpha</i> IFO 0195	121.60	54.00	1.65	0.20	DW	DW
88	<i>Pichia rhodanensis</i> IFO 1272	140.14	56.25	1.45	0.20	DW	DW
89	<i>Rhodotorula minuta</i> IFO 0387	231.00	138.60	1.16	0.11	W	C
90	<i>Rhodotorula rubra</i> IFO 0709	127.50	228.48	1.44	0.09	DW	C
91	<i>Saccharomyces carlsbergensis</i> IFO 0641	59.40	67.50	1.37	0.10	DW	D
92	<i>Saccharomyces rouxii</i> IFO 0487	71.71	58.50	1.39	0.11	D	D
93	<i>Saccharomyces sake</i> Kyoukai No.6	85.88	138.00	1.37	0.11	DW	W
94	<i>Saccharomycodes ludwigii</i> IFO 1043	217.08	132.00	1.46	0.14	DW	C
95	<i>Saccharomycopsis fibuligere</i> IFO1744	87.00	97.58	1.36	0.11	D	C
96	<i>Torula rubra</i> var. <i>alpha</i>	76.00	98.10	1.49	0.12	D	CD
97	<i>Torulospira delbueckii</i> IFO 0428	53.90	72.00	1.25	0.11	W	W
98	<i>Torulopsis aeria</i>	37.10	143.00	1.49	0.13	W	C
99	<i>Torulopsis candida</i> IFO 0768	109.08	164.25	1.42	0.09	W	C
100	<i>Trichosporon cutaneum</i> IFO 1198	216.25	222.60	1.24	0.11	W	C
101	Wine yeast	72.80	49.00	1.58	0.09	D	W
102	<i>Zygosaccharomyces rouxii</i> IFO 0505	134.32	89.25	1.15	0.16	DW	C

*, The clear zone was decided as follows : C, clear ; D, a little muddy ; W, muddy white ; CD, middle clear zone between C and D ; DW, middle clear zone between D and W

Table 3. Fibrinolytic activity of molds

Strain No.	Molds	Fibrinolytic activity mm ²		Protein (mg/ml)		Condition*	
		Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic
103	<i>Aspergillus niger</i> IFO 4414	871.20	266.00	1.42	1.20	C	DW
104	<i>Aspergillus oryzae</i> IFO 4176	752.90	358.00	1.52	1.20	C	D
105	<i>Aspergillus parasiticus</i> IFO 5241	414.52	321.10	1.33	1.29	C	C
106	<i>Monascus purpureus</i> IFO 4478	328.64	300.96	1.71	1.15	CW	DW
107	<i>Mucor circinelloides</i> f. <i>circinelloides</i> IFO 4554	0.00	254.10	1.63	1.19	C	DW
108	<i>Neurospora sitophila</i> IFO 4596	0.00	281.20	1.90	1.42	D	W
109	<i>Penicillium camembertii</i> IFO 5855	—	263.90	—	1.88	—	W
110	<i>Penicillium crysogenum</i>	0.00	325.60	1.86	1.46	C	W
111	<i>Rhizomucor pusillus</i> IFO 4578	0.00	326.80	1.10	1.37	C	DW
112	<i>Rhizopus javanicus</i> IFO 5441	684.48	642.60	1.08	2.04	CD	D
113	<i>Rhizopus oryzae</i> IFO 4706	342.38	221.97	1.12	1.39	W	W

*, The clear zone was decided as follows : C, clear ; D, a little muddy ; W, muddy white ; CD, middle clear zone between C and D ; DW, middle clear zone between D and W

Table 4. Fibrinolytic activity of basidiomycetes

Strain No.	Basidiomycetes	Fibrinolytic activity mm ²	Protein (mg/ml)	Condition*
C001	<i>Pleurotus ostreatus</i>	377.30	1.90	C
C003	<i>Flammulina velutipes</i>	119.85	1.92	C
C006	<i>Agaricus bisporus</i>	107.80	1.87	C
C007	<i>Agrocybe cylindracea</i>	327.52	1.70	C
C008-1	<i>Pleurotus cornucopiae</i>	187.50	1.76	C
C008-2	<i>Pleurotus cornucopiae</i>	584.00	1.59	C
C008-3	<i>Pleurotus cornucopiae</i>	676.40	1.54	C
C009	<i>Grifola frondosa</i>	284.00	2.03	C
C010	<i>Hypsizigus marmoreus</i>	216.05	1.94	C
C011	<i>Pleurotus sp.</i> (Awabitate)	630.00	1.62	C
	<i>Lentinus edodes</i>	306.29	1.90	C
	<i>Tricholoma matsutake</i>	419.58	1.69	C
W002	<i>Pleurotus ostreatus</i>	392.46	1.58	C
W003	<i>Collybia dryophila</i>	146.64	1.53	C
W008	<i>Laetitorus sulphureus</i>	225.00	1.63	C
W021	Mushroom Wild Type	177.00	1.79	C
W117	Mushroom Wild Type	162.41	1.65	C
W130	<i>Schizophyllum commune</i>	84.64	1.30	C
W351	<i>Pleurotus ostreatus</i>	441.00	1.53	C

*, The clear zone was decided as follows : C, clear ; D, a little muddy ; W, muddy white ; CD, middle clear zone between C and D ; DW, middle clear zone between D and W

In the case of bacteria, all strains except *Serratia plymuthica* IFO 3055 had fibrinolytic activity under aerobic or anaerobic conditions. Especially, *Brevibacterium sp.* P145 N. Kato, *Corynebacterium fascians* IAM 1079, and *Proteus vulgaris* IFO 3988 demonstrated high fibrinolytic activity under aerobic conditions, and *Alcaligenes faecalis* IAM B-141-1, *Erwinia arvideae* IFO 3830, *Klebsiella pneumoniae* IFO 12019, *Micrococcus luteus* IFO 3763, *Micrococcus lysodeikticus* Fleming IFO 3333 and *Serratia liquefaciens* IFO 12979 demonstrated high activity under anaerobic conditions. In the case of yeasts, all strains had fibrinolytic activity under both conditions. *Cryptococcus albidus* IFO 0378 demonstrated high activity of fibrinolytic enzymes when it was cultured under aerobic conditions. All strains of molds cultured under anaerobic conditions dissolved fibrin. On the other hand, in the case of basidiomycetes, fibrinolytic activity of *Pleurotus sp.* was higher, and the clear zone of all basidiomycetes showed "C" (clear). In this experiment, we found that almost all microorganisms had fibrinolytic activity. Therefore, fibrinolytic enzymes of many microorganisms and fermented foods produced

by them may be used to protect from thrombosis.

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