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Screening of Alcohol Dehydrogenase and Amylase of Microorganisms

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Twenty strains of bacteria and 10 strains of basidiomycetes cultured under aerobic conditions, and 3 strains of bacteria and 7 strains of yeasts cultured under anaerobic conditions demonstrated high alcohol dehydrogenase activity. Seven strains of bacteria and 4 strains of basidiomycetes cultured under aerobic conditions and 1 strain of yeast cultured under anaerobic conditions demonstrated high amylase activity.

Introduction

The earliest written records indicate that alcohol(ethyl alcohol)has been enjoyed by humans for thousands of years. It's microbial origin was only established 140 years ago through the work of Louis Pasteur[1]. Beer, wine and sake are the most common alcoholic beverages, and they are consumed by many people around the world. In general, *S. cerevisiae* has long been used for producing alcoholic beverages since it has an alcohol dehydrogenase[2]. Sake is an alcohol drink of a traditional fermented food in Japan. The fermentation of sake consists of simultaneous fermentation of both amylolysis by *Aspergillus oryzae* and alcohol fermentation by *Saccharomyces cerevisiae*. However, not only yeast but other microorganisms produce alcohol dehydrogenase, and they may be used to make some alcoholic beverages.

Therefore, we did a screening of alcohol dehydrogenase and amylase of various microorganisms.

Materials and Methods

Organisms

Sixty-eight strains of bacteria, 34 strains of yeasts, 11 strains of molds and 19 strains of basidiomycetes were used.

Medium and culture conditions

Bacteria were grown in 300ml Erlenmyer flasks with 100ml of the medium(pH 7.0)containing 0.5% meat extract, 0.5% NaCl and 1.0% peptone, at 28℃ for 24hr. Lactic acid bacteria were grown in 300ml Erlenmyer flasks with 100ml of the medium(pH 7.2)containing 0.24% meat extract, 0.5% yeast extract, 0.05% soluble starch, 1.0% protease peptone, 0.4% Na_2HPO_4, 0.05% glucose and 0.44% liver extract, at 37℃ for 24hr. Yeasts, molds and basidiomycetes were grown in 300ml Erlenmyer flasks with 100ml of the medium(pH 5.5～6.0) containing 2% malt extract at 28℃ for 24hr. Molds and basidiomycetes were grown in the medium at 25℃ for 2 weeks. Cultivation was carried out under aerobic conditions on a rotary shaker(100rpm)and under anaerobic conditions. After cultivation, the culture broth was

centrifuged(4℃, 20min, 8000rpm)to collect the cells. Molds and basidiomycetes were filtered with mesh. The cells were washed twice with 0.85% NaCl. The cells were stored at −20℃.

Preparation of cell free extract

The cells in 10mM potassium phosphate buffer(pH 7.0)were subjected to sonication with an ultrasonic oscillator(BRANSON, SONIFIER 250)15sec×4 times(bacteria, yeasts, molds)and 15sec×8～12 times(basidiomycetes)below 0～8 ℃. The undestroyed cells and debris were removed by centrifugation at 15,000rpm for 10min at 4℃. The supernatant solution obtained was used as the cell free extract.

Electrophoresis

Gel electrophoresis of the native enzyme was carried out with 7.5% polyacrylamide gel by the Davis[3] method. Twenty micrograms of ADH mixed with 20μl of 10mM potassium phosphate buffer(pH 7.0)or 20μl of cell free extract were electrophoresized at 20mA per gel until the dye front reached the bottom of the gel. After electrophoresis, the gel was stained with enzymatic activity staining mixture containing 0.2M Tris–HCl buffer(pH 8.5), 15mM NAD^+, 80mM ethanol, 6.5mM phenazine methosulfate and 12mM nitro blue tetrazolium.

Amylase activity

Step 1. Medium and cultivation condition of amylase master plates and starch plates : Bacteria were cultured on plates of 3.5% nutrient agar medium containing 0.5% agar(pH 7.0)at 28℃. Lactic acid bacteria were cultured on plates of 6.0% BL agar medium containing 0.5% agar(pH 7.2)at 37℃. Yeasts and molds were cultured on plates of 2% malt extract medium containing 2% agar(pH 5.6)at 28℃. The starch medium consisted of 5.0% sodium starch, 0.2% yeast extract and 2% agar. The medium of bacteria was adjusted to pH 7.5, and the medium of yeasts and molds was adjusted to pH 5.6.

Step 2. Amylase replica method : When colonies of microorganisms on the master plate were sufficiently grown, filter paper was put on the master plate and the colonies were transfered to the filter paper. The filter paper was put on starch plates to transfer colonies. Microorganisms on replica plates were cultured at 28℃ for 3 days under both aerobic and anaerobic conditions. Filter paper of 10% sodium iodic was put on the plate. And then, the coloring of microorganisms on the starch plate was checked.

Step 3. Measurement of amylase activity : The reaction mixture contained 2.5% of sodium starch, 5mM of $CaCl_2$(containing 50mM of acetic acid buffer)and cell free extract. Incubation was carried out at 40℃ for 30min with a test tube 16mm in diameter. The reaction was stopped with boiling water for 10min. Then, 0.5N acetic acid buffer and 0.1% sodium iodic were added to the reaction mixture. The reaction mixture was measured for absorbance at 700nm with a HITACHI 150–20 double beam spectrophotometer.

Results and Discussion

Polyacrylamide gel electrophoresis of the native enzyme followed by activity staining of alcohol dehydrogenase is shown in Fig. 1. Amylase activity of microorganisms on the starch plate is shown in Fig. 2. The activity staining of alcohol dehydrogenase and amylase activity of various microorganisms are summarized in Table 1, 2, 3 and 4.

Twenty(*Bacillus cereus* IFO 3001, *Bacillus megaterrium* NI 8100 NTH B12, *Bacillus sphaericus* IFO 3525, *Bacillus brevis* IFO 3007 , *Bacterium ketoglutamicus* SO(1)Shionogi Co., Ltd, *Bacterium orleanense* IFO 3259, *Brevibacterium sp.* P145 Phage host, *Corynebacterium glutamicum* No.534 ATCC 13032, *Corynebacterium pseudodiphteritium*, *Enterobacter aerogenes* IFO 3320, *Enterobacter cloacae* IAM 1221, *Erwinia arvideae* IFO 3830, *Erwinia carotovora* IFO 3380, *Klebsiella pneumoniae* IFO 3317, *Pseudomonas fluorescens* IFO 3081, *Pseudomonas graveolens* IFO 3460, *Pseudomonas iodium* IFO 3558, *Pseudomonas solanacearum* IFO 3509, *Pseudomonas striafaciens* IFO 3309, *Serratia liquefaciens* IFO 12979)strains of bacteria

and 10(C003 *Flammulina velutips*, C007 *Agrocybe cylindracea*, C008-1 *Pleurotus cornucopiae*, C008-2 *Pleurotus cornucopiae*, C008-3 *Pleurotus cornucopiae*, C009 *Grifola frondosa*, C010 *Hypsizigus marmoreus*, C011 *Pleurotus sp.* (Awabitake), *Lentinus edodes*, W008 *Laetitorus sulphureus*)strains of basidiomycetes cultured under aerobic conditions, and 3(*Klebsiella pneumoniae* IFO 12019, *Proteus morganii* IFO 3168, *Staphylococcus epidermidis* IFO 3762)strains of bacteria and 7(Awamori yeast(Sakamoto), *Candida pelliculosa* IFO 0707, *Hansenula miso* IFO 0146, *Pichia anomala* IFO 0568, *Pichia polymorpha* IFO 0195, *Pichia rhodanensis* IFO 1272, *Saccharomyces sake* Kyoukai No.6)strains of yeasts cultured under anaerobic conditions demonstrated high alcohol dehydrogenase activity.

Seven(*Bacterium cadaveris* IFO 3731, *Bacterium ketoglutamicus* SO(1)Shionogi Co., Ltd, *Bacterium orleanense* IFO 3259, *Enterobacter aerogenes* IFO 3320, *Erwinia carotovora* IFO 3380, *Klebsiella pneumoniae* IFO 3317, *Pseudomonas fluorescens* IFO 3081)strains of bacteria, 4(*Aspergillus oryzae* IFO 4176, *Monascus purpureus* IFO 4478, *Rhizomucor pusillus* IFO 4578, *Rhizopus javanicus* IFO 5441)strains of molds and 4(C003 *Flammulina velutips*, C010 *Hypsizigus marmoreus*, C011 *Pleurotus sp.* (Awabitake), W117 Mushroom Wild Type) strains of basidiomycetes cultured under aerobic conditions and 1(Wine yeast)strain of yeast cultured under anaerobic conditions demonstrated high amylase activity.

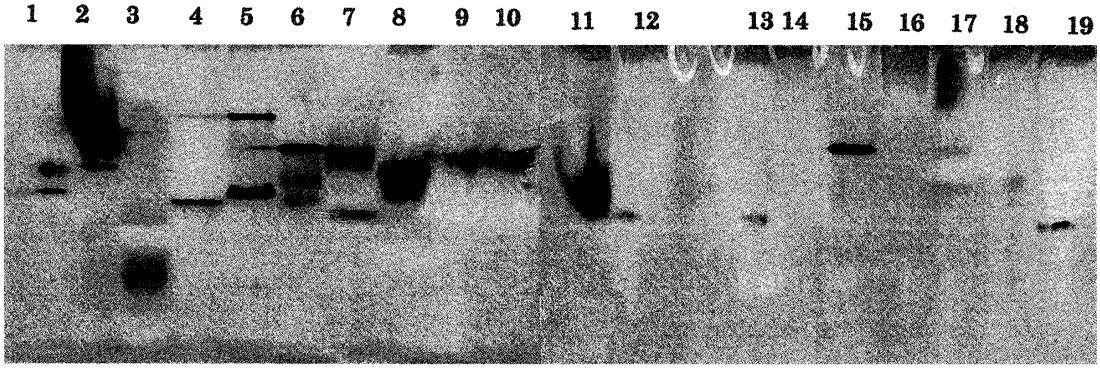

Fig. 1. Activity staining.

The activity staining of alcohol dehydrogenase was examined by the method described in Materials and Methods. 1, *Pleurotus ostreatus*(C001): 2, *Flammulina velutips* : 3, *Agaricus bisporus* : 4, *Agrocybe cylindracea* : 5, *Pleurotus cornucopiae*(C008-1): 6, *Grifola frondosa* : 7, *Hypsizigus marmoreus* : 8, *Pleurotus sp.* (Awabitake): 9, *Pleurotus cornucpiae*(C008-2): 10, *Pleurotus cornucopiae*(C008-3): 11, *Lentinus edodes* : 12, *Tricholoma matsutake* : 13, *Pleurotus ostreatus*(W002): 14, *Collybia dryophila* : 15, *Laetitorus sulphureus* : 16, Mushroom Wild Type(W021): 17, Mushroom Wild Type(W117): 18, *Schizophyllum commune* : 19, *Pleurotus ostreatus*(W351)

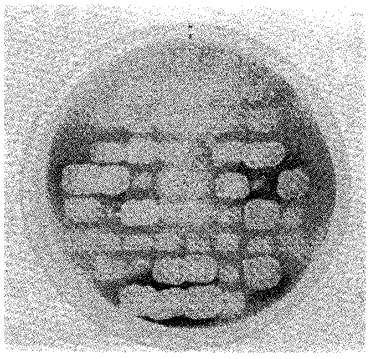

Fig. 2. Amylase activities of microorganisms on the starch plate.

Table 1. Activities of alcohol dehydrogenase and amylase of bacteria

Strain No.	Bacteria	ADH activity*		Amylase activity**	
		Aerobic	Anaerobic	Aerobic	Anaerobic
1	*Agrobacterium radiobacter* IAM 1526	+	+	0.235	0.260
2	*Agrobacterium tumefaciens* IAM B-26-1	+	+	0.279	0.300
3	*Alcaligenes faecalis* IAM B-141-1	+	+	0.273	0.232
4	*Alcaligenes polymorpha*	+	+	0.232	0.288
5	*Arthrobacter globiformus* IFO 12137	+	+	0.225	0.285
6	*Arthrobacter oxydans* IFO 12138	+	+	0.253	0.263
7	*Arthrobacter pascens* IFO 12139	+	+	0.272	0.260
8	*Arthrobacter simplex* IFO 3530	+	+	0.266	0.244
9	*Arthrobacter ureafaciens* IFO 12140	+	+	0.267	0.230
10	*Bacillus brevis* IFO 3331	+	+	0.278	0.277
11	*Bacillus cereus* IFO 3001	+ +	−	0.252	0.298
12	*Bacillus megaterrium* NI 8100 NTH B12	+ +	+	0.265	0.280
13	*Bacillus pumilus* IFO 3030	+	+	0.314	0.266
14	*Bacillus sphaericus* IFO 3525	+ +	+	0.218	0.275
15	*Bacillus brevis* IFO 3007	+ +	+	0.332	0.269
16	*Bacterium cadaveris* IFO 3731	+	+	0.081	0.232
17	*Bacterium ketoglutamicus* SO(1)Shionogi Co., Ltd	+ +	+	0.082	0.247
18	*Bacterium orleanense* IFO 3259	+ +	+	0.057	0.232
19	*Brevibacterium sp.* P145 Phage host	+ +	+	0.192	0.233
20	*Brevibacterium sp.* P145 N.Kato	+	+	0.193	0.148
21	*Corynebacterium fascians* IAM 1079	+	+	0.238	0.235
22	*Corynebacterium glutamicum* No.534 ATCC 13032	+ +	+	0.118	0.246
23	*Corynebacterium pseudodiphteritium*	+ +	+	0.186	0.221
24	*Enterobacter aerogenes* IFO 3320	+ +	+	0.053	0.228
25	*Enterobacter cloacae* IAM 1221	+ +	+	0.214	0.239
26	*Erwinia arvideae* IFO 3830	+ +	+	0.297	0.259
27	*Erwinia carotovora* IFO3380	+ +	+	0.087	0.246
28	*Escherichia coli* K12 IFO 3208	+	+	0.223	0.235
29	*Escherichia freundii* S-96	+	+	0.260	0.126
30	*Escherichia intermedia* A-21	+	+	0.239	0.260
31	*Klebsiella pneumoniae* IFO 3317	+ +	+	0.018	0.241
32	*Klebsiella pneumoniae* IFO 12009	+	+	0.249	0.160
33	*Klebsiella pneumoniae* IFO 12019	+	+ +	0.271	0.201
34	*Klebsiella pneumoniae* IFO 12932	+	+	0.278	0.251
35	*Klebsiella pneumoniae* IFO 13541	+	+	0.262	0.248
36	*Klebsiella pneumoniae* IFO 13703	+	+	0.229	0.245
37	*Micrococcus luteus* IFO 3763	+	+	0.247	0.194
38	*Micrococcus lysodeikticus Fleming* IFO 3333	+	+	0.260	0.231
39	*Micrococcus roseus* IFO 3764	+	+	0.263	0.225
40	*Micrococcus rubens* IFO 3768	+	+	0.252	0.214
41	*Proteus mirabilis* IFO 3849	+	+	0.260	0.308
42	*Proteus morganii* IFO 3168	+	+ +	0.217	0.209
43	*Proteus vulgaris* IFO 3988	+	+	0.290	0.214
44	*Pseudomonas fluorescens* IFO 3081	+ +	+	0.062	0.219
45	*Pseudomonas fragi* IFO 3458	−	+	0.239	0.245
46	*Pseudomonas graveolens* IFO 3460	+ +	+	0.257	0.282
47	*Pseudomonas iodium* IFO 3558	+ +	+	0.246	0.293
48	*Pseudomonas solanacearum* IFO 3509	+ +	+	0.280	0.290
49	*Pseudomonas striafaciens* IFO 3309	+ +	+	0.252	0.287
50	*Sarcina aurantiaca* IFO 3064	+	+	0.256	0.309
51	*Sarcina lutea* IFO 1099	+	+	0.283	0.266
52	*Serratia liquefaciens* IFO 12979	+ +	+	0.235	0.273
53	*Serratia marcescens* IFO 3054	+	+	0.249	0.270
54	*Serratia plymuthica* IFO 3055	+	+	0.270	0.271
55	*Staphylococcus aureus* IFO 3060	+	−	0.264	0.296
56	*Staphylococcus epidermidis* IFO 3762	+	+ +	0.276	0.275
57	*Bifidobacterium adolescentis* M101-4	−	−	0.213	0.268
58	*Bifidobacterium bifidum* A234-4	−	−	0.192	0.296
59	*Bifidobacterium breve* I-53-8	−	−	0.210	0.279
60	*Bifidobacterium infantis* I-10-5	−	−	0.184	0.266
61	*Bifidobacterium longum* M101-2	−	−	0.185	0.297
62	*Lactobacillus bulgaricus* IFO 13953	−	−	0.190	0.253
63	*Lactobacillus clerbueckii* IFO 3202	−	−	0.226	0.250
64	*Lactobacillus paracasei* IFO 3953	−	−	0.215	0.311
65	*Lactococcus cremoris* IFO 3427	−	−	0.206	0.281
66	*Lactococcus lactis* IFO 12007	−	−	0.212	0.211
67	*Pediococcus acidilactici* IFO 3888	−	−	−	0.251
68	*Streptococcus thermophilus* IFO 13957	−	+	0.232	0.283

*, Alcohol dehydrogenase(ADH) + +, higher; +, nomal; −, no activity.
**, Measurement by absorbance at 700nm.

Table 2. Activities of alcohol dehydrogenase and amylase of yeasts

Strain No.	Yeasts	ADH activity*		Amylase activity**	
		Aerobic	Anaerobic	Aerobic	Anaerobic
69	Awamori yeast (Sakamoto)	+	+ +	0.275	0.279
70	*Candida guilliermondii* IFO 0566	−	−	0.278	0.293
71	*Candida pelliculosa* IFO 0707	+	+ +	0.284	0.280
72	*Candida utilis* IFO 0619	−	−	0.279	0.283
73	*Cryptococcus albidus* IFO 0378	−	−	0.271	0.290
74	*Cryptococcus laurentii* IFO 0609	−	−	0.265	0.288
75	*Cryptococcus neoformans* IFO 0410	−	−	0.159	0.300
76	*Debaryomyces japonicus* IFO 0039	−	−	0.209	0.262
77	*Debaryomyces vini* Y.U.	−	−	0.210	0.289
78	*Endomycopsis capsularis* IFO 0672	−	−	0.185	0.230
79	*Endomyces decipiens* IFO 0102	+	−	0.174	0.263
80	*Hansenula miso* IFO 0146	+	+ +	0.195	0.257
81	*Hansenula suaveolens* IFO 0992	−	−	0.256	0.274
82	*Hansenula wingei* IFO 0976	−	−	0.278	0.292
83	*Kloeckere apiculate* IFO 0865	−	−	0.288	0.284
84	*Nadsonia fulvescens* IFO 0666	−	−	0.185	0.271
85	*Pichia anomala* IFO 0568	+	+ +	0.180	0.274
86	*Pichia orientalis* IFO 1279	−	−	0.246	0.148
87	*Pichia polymorpha* IFO 0195	+	+ +	0.262	0.226
88	*Pichia rhodanensis* IFO 1272	+	+ +	0.279	0.264
89	*Rhodotorula minuta* IFO 0387	−	−	0.298	0.301
90	*Rhodotorula rubra* IFO 0709	−	−	0.268	0.294
91	*Saccharomyces carlsbergensis* IFO 0641	−	+	0.282	0.265
92	*Saccharomyces rouxii* IFO 0487	−	−	0.247	0.205
93	*Saccharomyces sake* Kyoukai No.6	+	+ +	0.284	0.265
94	*Saccharomycodes ludwigii* IFO 1043	−	−	0.287	0.252
95	*Saccharomycopsis fibuligere* IFO1744	−	−	0.225	0.231
96	*Torula rubra var. alpha*	−	−	0.287	0.288
97	*Torulaspora delbueckii* IFO 0428	−	−	0.258	0.243
98	*Torulopsis aeria*	−	−	0.260	0.294
99	*Torulopsis candida* IFO 0768	−	−	0.256	0.298
100	*Trichosporon cutaneum* IFO 1198	−	−	0.293	0.298
101	Wine yeast	−	−	0.276	0.006
102	*Zygosaccharomyces rouxii* IFO 0505	−	−	0.302	0.234

*, Alcohol dehydrogenase (ADH) + +, higher; +, nomal; −, no activity.
**, Measurement by absorbance at 700nm.

Table 3. Activities of alcohol dehydrogenase and amylase of molds

Strain No.	Molds	ADH activity*		Amylase activity**	
		Aerobic	Anaerobic	Aerobic	Anaerobic
103	*Aspergillus niger* IFO 4414	−	−	0.240	0.304
104	*Aspergillus oryzae* IFO 4176	−	−	0.023	0.309
105	*Aspergillus parasiticus* IFO 5241	−	−	0.249	0.279
106	*Monascus purpureus* IFO 4478	−	−	0.018	0.290
107	*Mucor circinelloides f. circinelloides* IFO 4554	−	−	0.179	0.291
108	*Neurospora sitophila* IFO 4596	−	−	0.271	0.276
109	*Penicillium camembertii* IFO 5855	−	−	−	0.293
110	*Penicillium crysogenum*	−	−	0.162	0.282
111	*Rhizomucor pusillus* IFO 4578	−	−	0.029	0.266
112	*Rhizopus javanicus* IFO 5441	−	−	0.068	0.280
113	*Rhizopus oryzae* IFO 4706	+	−	0.165	0.219

*, Alcohol dehydrogenase (ADH) + +, higher; +, nomal; −, no activity.
**, Measurement by absorbance at 700nm.

Table 4. Activities of alcohol dehydrogenase and amylase of basidiomycetes

Strain No.	Basidiomycetes	ADH activity*	Amylase activity**
C001	*Pleurotus ostreatus*	+	0.199
C003	*Flammulina velutips*	++	0.049
C006	*Agaricus bisporus*	+	0.204
C007	*Agrocybe cylindracea*	++	0.154
C008-1	*Pleurotus cornucopiae*	++	0.156
C008-2	*Pleurotus cornucopiae*	++	0.195
C008-3	*Pleurotus cornucopiae*	++	0.190
C009	*Grifola frondosa*	++	0.165
C010	*Hypsizigus marmoreus*	++	0.091
C011	*Pleurotus sp.* (Awabitake)	++	0.086
	Lentinus edodes	++	0.216
	Tricholoma matsutake	+	0.148
W002	*Pleurotus ostreatus*	+	0.215
W003	*Collybia dryophila*	−	0.242
W008	*Laetitorus sulphureus*	++	0.146
W021	Mushroom Wild Type	−	0.268
W117	Mushroom Wild Type	+	0.075
W130	*Schizophyllum commune*	+	0.227
W351	*Pleurotus ostreatus*	+	0.213

*, Alcohol dehydrogenase(ADH) ++, higher; +, nomal; −, no activity.
**, Measurement by absorbance at 700nm.
The activities of alcohol dehydrogenase and amylase of basidiomycetes were examined with cells obtained under aerobic conditions.

References

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