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Growth of Yesasts in Extracts from Coniferous Barks

——Studies on Strains Tolerable to Tannin and Solvents to Extract Fermentative Sugars Effectively——

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1. Introduction

This study is a basical one for utilization of fermetative sugars in wood bark extract. Japanese forests produce much amounts of barks with timbers. Almost of all barks are discarded without direct burning for energy production because of economical reasons. As barks have much extracts, including fermentative sugars, their use by yeasts will add an economical value to bark. Water extraction is the most simple method of obtaining fermentative sugars but water extract always contains tannin with sugars[1]. As tannin usually inactivats enzymes[2, 3] and inhibits growth of microorganism[4, 5], these authors tried to select the yeasts to be tolerable to tannin in this study firstly and then investigated the best condition to get fermentative sugars from bark.

2. Experiment

All schedules of experiments were made under consideration to be possibly simple because of energy saving. Evaporation was avoided from begining to end. Thirty yeasts were supplied by Institute of the Applied Microbiology of the University of Tokyo and Kyowa Fermentation Industry Co., Ltd. as shown in Table 1.

2.1 Selection of yeasts under aerobic condition

The basal mediums were shown in footnotes of Table 3. Catechim was added to be 0.2% of the basal medium. When yeasts were incubated at 26.5℃, their growth stopped after 13 -14 hours and then amounts of the yeasts were evaluated after 19 hours by an absortion of 660 nm of UV spectrum. Amounts of sugars were measured by method of phenol-surfuric acid.

2.2 Selection of yeasts under anaerobic conditions

Basal medium contains 1% of sugar and 0.25% of commertial yeast extract. Catechin was added to be 0.2% of the basal medium. Yeasts were incubated without shaking. Production of ethanol was checked by Durham test. That is, yeasts were incubated for 1-7 days to make a given volume of CO_2 gas in a small tube dipped in the medium. The numbers of days were counted as an indicator showing production of ethanol.

2.3 Studying of extracting condition to yield the most amounts of fermentative sugars

Five extracting conditions were examined by the scheme shown in Figure 1 on yield of fermentative sugars. Ratio of barks to solvents was always 10 : 1 (w/w). The obtained solutions were never concentrated. When acid was used for extraction, acidic solutions were neutralized by solid barium hydroxide and the obtained supernatant was separated by a centrifuge.

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Table 1. Tested yeasts

No. of strain	Name
1	*Saccharomyces cerevisiae* RIB 6001
2	*S. cerevisiae* RIB 6002
3	*S. cerevisiae* IAM 4274
4	*S. cerevisiae* RIB 6852
5	*Candida lusitaniae* IAM 12189
6	*Hansenula saturnus* IAM 12217
7	*Candida boidinii* IAM 12269
8	*C. intermedia* IAM 12185
9	*C. kefyr* IAM 12195
10	*C. maltosa* IAM 12247
11	*Debaryomyces castellii* IAM 4977
12	*Hansenula fabianii* IAM 12212
13	*Kluyveromyces marxianus* IAM 4985
14	*Candida maltosa* IAM 12248
15	*C. mogii* IAM 4979
16	*C. parapsilosis* IAM 12192
17	*C. pseudotropicalis* IAM 4829
18	*C. utilis* IAM 4961
19	*Saccharomyces cerevisiae* IAM 4125
20	*S. cerevisiae* IAM 4512
21	*Schizosaccharomyces japonicus* IAM 12257
22	*S. pombe* IAM 4863
23	*Wickerhamia fluorescens* IAM 4971
24	*Saccharomyces cerevisiae* ATCC 26018
25	*S. cerevisiae* ATCC 26603
26	*S. diastaticus* IFO 1046
27	*S. sake* IFO 476
28	*S. uvarum* ATCC 26602
29	*Schizosaccharomyces pombe* ATCC 2476
30	*Jorulopsis glabrata* IFO 622

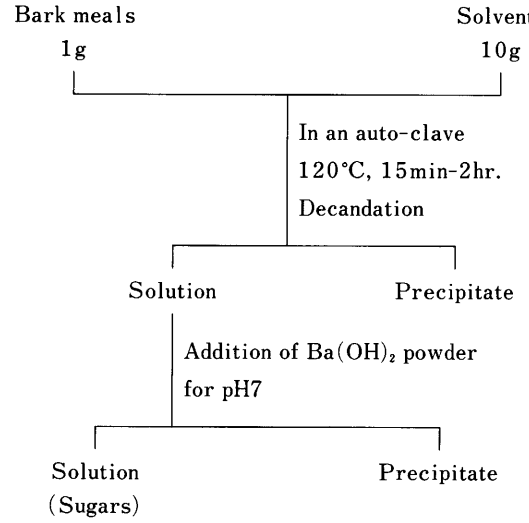

Fig. 1. An extracting process.

Table 2. Effects of catechin in growth of yeasts

No. of strain	(%) by yeasts*1	(%) by sugars*2
1	84	113
2	87	113
3	69	113
4	29	113
5	83	102
6	91	102
7	37	54
8	86	94
9	67	25
10	88	97
11	124	433
12	76	113
13	89	144
14	88	137
15	90	135
16	93	136
17	58	135
18	93	155
19	89	156
20	108	(−)
21	106	107
22	47	104
23	116	125
24	98	103
25	97	106
26	100	106
27	74	2105
28	114	107
29	191	141

*1 $\frac{\text{Amounts of produced yeasts in catechin medium}}{\text{Amounts of produced yeasts in basal medium}} \times 100.$

*2 $\frac{\text{Consumed sugar in catechin medium}}{\text{Consumed sugar in basal medium}} \times 100.$

Legend: See Table 3 on basal medium.

3. Result and Discussion

3.1 Selection of yeasts under aerobic conditions

Thirty yeasts were incubated in the basal medium and in the basal medium with catechin, and their growth was evaluated by produced yeasts and by consumed sugars. The results were shown in Table 2. It was shown that half of the tested yeasts grew without influence of catechin addition and then six yeasts were selected for the following experiments. The selected six yeasts were incubated in the basal medium containing sugi extract instead of glucose. The results were compared with ones in glucose medium, as shown in Table 3. It was shown that four yeasts (No. 2, 4, 10 and 18) grew well in the bark extract medium.

3.2 Selection of yeasts under anaerobic conditions

Thirty yeasts were anaerobically incubated in the basal medium and in the catechin

Table 3. Effects of bark extracts in aerobic growth of yeasts

No. of strain	(%) by yeasts*1		(%) by sugars*2	
	Sugi	Hinoki	Sugi	Hinoki
2	71	91	87	115
4	71	91	86	107
10	86	119	86	98
18	88	112	86	111
29	130	300	118	31
30	74	110	80	89

*1 See foot note (*1) of Table 2. Set "Bark extracts" in stead of "Catechin".

*2 See foot note (*2) of Table 2. Set "Bark extracts" in stead of "Catechin".

Legend: Basal medium contains 0.2% of $(NH_4)_2SO_4$, 0.05% of $MgSO_4 \cdot 7H_2O$, 0.2% of $(NH_4)_2CO_3$, 0.2% of KH_2PO_4 and 0.3% of commercial yeast extract. Amounts of bark extracts were prepared for their fermentative sugars to be 1% in the basal medium. The bark extracts contain following sugars and phenols (% to oven-dried bark).

	Sugars	Phenols
Sugi	0.76%	0.61%
Hinoki	0.73%	0.37%

Table 4. Influence of catechin on anaerobic growth of yeasts ——Days spent by yeasts for production of gas having a given volume

No. of strain	In basal medium	In medium with catechin
1, 4	1	1
2, 26	1	2
3	1	1
5, 11	7	L7
6, 12, 14, 17	3	L3
8, 13, 16, 28	3	M4
9, 10, 18, 19, 22	2	M3
20, 21, 24	2	L2
23	3	M4
25	2	L2
27	3	L3
29	4	L4
30	4	M5

Legend: L, less than; M, more than.
See Table 3 on basal medium.

containing medium. Numbers of days, which yeasts spent producing a given volume of CO_2 gas, were counted, as shown in Table 4. It was shown that eleven yeasts were not influenced by addition of catechin. Then the gas production by the eleven yeasts was

Table 5. Influcence of different sugars on anaerobic growth of yeasts——Days spent by yeasts for production of gas having a given volume

No. of strain	Glucose		Fructose		Sucrose	
	Basal	Catechin	Basal	Catechin	Basal	Catechin
1	1	1	1	2	1	1
4	2	1	1	2	2	1
6	2	3	2	2	2	2
12	2	2	2	2	2	2
14	2	2	3	3	3	3
17	2	2	2	2	2	2
20	2	1	2	2	2	2
21	2	1	2	2	2	2
23	3	2	3	3	3	3
24	1	1	2	2	1	2
29	2	2	2	2	2	2

Legend: "Basal" means "Basal medium", containing 1% of a sugar, 0.5% of commercial yeast extract and 0.25% of pepton. "Catechin" means "Catechin containing medium" (sugar : catechin=5 : 1 (w/w)).

Table 6. Influence of a sugi extract on anaerobic growth of yeasts——Days spent by yeasts for production of gas having a given volume

No. of strain	Basal medium	Sugi extract medium
1	1	2
4	2	2
6	2	4
12	2	4
14	3	4
17	2	4
20	2	2
21	2	2
23	2	2
24	2	2
29	2	2

Legend: Sugi extract contains 3.5% of sugars, including 0.9% of glucose, 0.3% of fructose and 0.1% of sucrose, and 2.5% of phenol (% of oven dried bark).

investigated on influence of three kinds of sugars (glucose, fructose and sucrose) to show similar tendency, as shown in Table 5. Finally, the eleven yeasts were examined on gas production in sugi extract medium, instead of sugar and catechin medium. All of the yeasts produced gas normally without influence of bark tannin, as shown in Table 6. Therefore it was shown that the selected yeasts were suitable for production of ethanol in sugi extract medium.

3.3 Studying of extracting conditions from sugi bark on fermentative sugars

Our repeated experiments showed that hot water extraction do not make enough fermentative sugars in solution for growth of yeasts at all times. Then another conditions

Table 7. Sugar and phenol contents of sugi extracts by several conditions (% of solvents)

Solvent	Time of extraction	Inner bark		Outer bark	
		Sugar	Phenol	Sugar	Phenol
Water	60 min	2.24	1.20	0.27	0.07
1% H_2SO_4	60 min	2.70	0.57	0.92	0.06
1% H_2SO_4	30 min	3.13	0.79	0.68	0.05
1% H_2SO_4	15 min	3.39	0.91	0.62	0.04
2% H_2SO_4	60 min	1.20	–	–	–

Legend: Barks were extracted in an auto-clave.

Table 8. Ethanol produced from sugi extracts by yeasts (% of the medium)

No. of strain	Inner bark Days after inoculation			Outer bark		
	1st	2nd	3rd	1st	2nd	3rd
4	1.00	0.96	0.82	0.00	0.00	0.00
12	0.63	0.68	0.66	0.00	0.00	0.00
17	0.89	0.87	0.84	0.06	0.03	0.01
20	0.87	0.96	0.99	0.08	0.16	0.06
21	0.04	0.98	1.13	0.09	0.07	0.10
23	0.80	0.87	0.79	0.12	0.09	0.09
24	0.89	0.89	0.79	0.08	0.17	0.19
29	0.59	1.15	1.17	0.10	0.14	0.10

Table 9. Monomeric sugars content of 1% H_2SO_4 extracts from sugi bark (% of solvents)

Sugar	Inner bark	Outer bark
Glucose	1.38	0.20
Others	0.65	0.15

Note: See footnotes of Table 7.

for extraction were investigated.

Sugi barks were extracted under 5 kinds of extracting conditions in an auto-clave and the extracts were examined on sugars and phenol contents. The results were shown in Table 7. The most amounts of sugars were obtained by extraction of inner barks with 1% H_2SO_4 for 15 min and of outer barks with 1% H_2SO_4 for 60 min, respectively. The extracts were used for media of fermentation with 0.25% of commercial yeasts extracts. The amounts of produced ethanol were observed, as shown in Table 8. Six of the yeasts produced 0.8–1.0% of ethanol in the medium of inner bark extracts at the first day, and two (No. 21 and 29) of them could not do it at the day but did at the 3rd day. All of the yeasts produced 0.01–0.12% of ethanol in the medium of outer bark extracts.

In order to know fermentative sugars in the extracts, monosaccharides were measured. The reslts were shown in Table 9. The inner bark extract contains 1.38% of glucose. As

Table 10. Sugar content of pines extracts (% of solvents)

Solvent	Akamatsu	Ezomatsu	Karamatsu	Todomatsu	Benimatsu
Water	0.46	0.36	0.83	0.21	0.64
1% H_2SO_4	1.00	0.68	1.20	0.83	1.06

Note: See footnotes of Table 7.

Table 11. Monomeric sugar contents of 1% H_2SO_4 extracts of pines (% of solvents)

Name of sugar	Akamatsu	Ezomatsu	Karamatsu	Todomatsu	Benimatsu
Glucose	0.13	0.14	0.31	0.08	0.32
Arabinose	0.52	0.40	0.27	0.26	0.48
Xylose	0.04	0.02	0.01	0.03	0.04

Note: See footnotes of Table 7.

Table 12. Ethanol produced from pines extracts by yeasts (% of the medium)

No. of strain	Akamatsu	Ezomatsu	Karamatasu	Todomatsu	Benimatsu
4	0.08	0.08	0.13	0.06	0.11
12	0.03	0.04	0.17	0.05	0.13
17	0.13	0.13	0.06	0.09	0.14
20	0.04	0.09	0.19	0.05	0.14
21	0.01	0.04	0.06	0.04	0.04
23	0.08	0.11	0.15	0.06	0.13
24	0.04	0.09	0.18	0.05	0.14
29	0.03	0.11	0.23	0.06	0.15

Note: See footnotes of Table 7.

those yeasts can produce 0.5% of ethanol from 1% of glucose in the basal medium, as shown in later, the production of 0.8–1% of ethanol in the media of inner bark extracts will depend on glucose mainly and on other sugars partly. The outer bark extract contains 0.20% of glucose. The fact is supposed to cause the poor production of ethanol in the media of outer bark extracts.

3.4 Studying of extracting condition from pines barks on fermentative sugars

The extracting condition of sugars was investigated in whole barks of pines to get more amount of sugars. Hot water and 1% sulfuric acid were used for solvents. The most amounts were obtained by extraction of barks with 1% of surfuric acid in an auto-clave, as shown in Table 10. Monomeric sugars were measured in the extracts also. The results were shown in Table 11. Arabinose was a main sugar in almost all of the extracts. The yeasts were incubated in the bark extracts. Concentration of produced ethanol in media were observed, as shown in Table 12. Less ethanol relatively to sugi extracts were produced in pines extracts. Fermentation of arabinose by the yeasts was examined and found to be impossible, as shown in Table 13. Therefore, the almost of all ethanol produced in pine extracts media was supposed to be in debt to glucose.

Table 13. Ethanol produced by yeasts in basal medium including glucose or arabinose (% of the medium)

No. of strain	1% Glucose	1% Arabinose
4	0.48	0.00
12	0.28	0.01
17	0.52	0.01
20	0.52	0.01
21	0.06	0.03
23	0.48	0.00
24	0.46	0.00
29	0.14	0.00

Leagend: Ethanol concentration in media after one day incubatione of yeasts.

In this experiments, some strains tolerable to tannin were selected and could grow in bark extracts. Those strains will possibly grow in tannin containing medium other than sugi bark extract (e.g. hinoki) and then a very simple method was established in utilization of fermentative sugars. However, this utility is limited because of shortage of fermentative sugars in bark extracts, though it was shown in pines bark extracts.

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Summary

Sugars were intended to be extracted from coniferous barks and used for growth of yeasts. As water extracts usually contain both sugars and tannin,30 yeasts were investigated on growing in medium cotaining phenols (tannin). It was shown that 4 yeasts aerobically and 11 yeasts anaerobically grew well in the media containing sugi bark extracts, respectively.

Several conditions were examined to dissolve more fermentative sugars from sugi and pines barks. The best ones were found for the extraction from sugi inner and outer, and pines barks, separately.

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針葉樹樹皮抽出物での酵母の生育

——タンニン耐性株および資化糖抽出溶剤に関する研究——

善本知孝・佐分義正

摘　　要

針葉樹々皮抽出物に含まれる糖を酵母の生育に使うのを意図した。水抽出物は糖のほかに常にタンニンを含み，そのため酵母は生育を阻害される。そこで先ずタンニンに耐性のある株を30株について追究し，好気條件で4株が，嫌気條件で11株が良好な生育をスギ抽出物培地で行うのを究明した。

温水抽出より多くの醱酵可能な糖をうる抽出方法について数種を検討し，スギ内，外皮，マツ樹皮夫々に適したものを見出した。