Selection of Mutants of *Corticium rolfsii* with Better Saccharification Activity on Raw Starch

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Resumé

In order to develop a raw starch saccharification process, mutants of *Corticium rolfsii* were isolated after irradiation of ultraviolet light on its protoplasts. Amylolytic activity of the mutants was examined and strains with higher activity were obtained.

Foreword

Glucose production on commercial scale has been mainly done by gelatinization of starch slurry by cooking and subsequent hydrolysis with amylase. This process requires a large amount of heat energy and the slurry at high concentration is not easily processed because of the viscous nature of the gelatinized starch.

Recently, several papers concerned with the direct saccharification of raw starch have been published. For the realization of this process, a new enzyme, which can hydrolyze uncooked starch at high temperature and low pH, must be obtained. In these reports, some microorganisms including *Aspergillus* sp.[1)2)3)4)], *Rhizopus* sp.[5)], *Bacillus circulans*[6)7)], *Streptococcus bovis*[8)9)] and *Chalara* sp.[10)11)], have been studied as inexpensive resources of the enzyme. However, the hydrolytic activity of their enzymes was not sufficient for this purpose, because they were not active at high starch concentration or at low pH.

Corticium rolfsii is known to produce a significant enzyme that hydrolyzes raw starch at low pH (Kaji et al.[12)13)14)]). Thus, this enzyme is very promising for glucose production from raw starch on industrial scale (Takao et al[15)]. and Sasaki.[16)]). It bears deep brown sclerotia but does not differentiate into fruiting body. In this study, we isolated some mutants of *C. rolfsii* which showed high enzyme activity than that of the parent

strain.

Experimental

1. Microorganism

Corticium rolfsii AHU 9627 was used in this study.

2. Culture media and maintenance of stock culture.

1) Components of culture media.

a) Oat meal stock culture medium: Thirty grams of oat meal was extracted with 1,000ml of hot water for 30min and the extract was filtered through gauze. Yeast extract (1.4g) and agar (10.5g) were added to the extract (700ml).

b) Seed culture medium: Polypepton (1.0g), yeast extract (0.2g), soluble starch (2.0g), NH_4NO_3 (0.18g) and $MgSO_4 \cdot 7H_2O$ (0.03g) were dissolved in tap water and filled up to 100ml.

The pH of the solution was adjusted to 5.0 with 1N-HCl.

c) Regeneration medium: Glucose (2.0g), yeast extract (0.1g), polypepton (0.15 g), $MgSO_4 \cdot 7H_2O$ (0.03g), sucrose (13.7g) and agar (final 2% for underlayer and 0.5% for upperlayer) were dissolved in 90ml of tap water. The pH of the medium was adjusted to 6.0 with 1N-HCl.

d) Assay medium for mutants: polypepton (3.0g), yeast extract (0.2g), soluble starch (2.0g), NH_4NO_3 (0.18g), $MgSO_4 \cdot 7H_2O$ (0.03g) and Tween 80 (0.1g) were dissolved in 100ml of tap water.

The pH of the medium was adjusted to 5.0.

e) Sterilization of the media: Every medium described above was autoclaved at 1.25 kg/cm^2 for 20min. Hot oat meal medium was stirred with a magnetic stirrer to disperse the precipitates, and the slants were prepared.

2) Maintenance of stock culture.

Oat meal agar slants were used for the stock culture. After incubation at 30°C for 7-10 days, deep brown sclerotia were formed. The slant cultures were stored at 4°C. Once in a month they were transplanted to new media.

3) Assay of glucoamylase activity.

Soluble starch (0.56g as dry weight) was dissolved in about 80ml of water and

boiled, cooled down to room temperature, and filled up to 100ml with 5ml of 1M acetate buffer (pH 4.5) and water. A test tube containing 0.9ml of the starch solution and 0.1ml of the eyzyme solution was incubated at 40°C for 30min. Three mililiters of DNS reagent was added to the tube, and the quantity of glucose formed was determined. One unit (GU/ml) of the enzyme activity was defined as the formation of 1mg/ml glucose in the solution under the above conditions.

4) Determination of the reducing sugar with DNS.

To 1,500ml of 4.5% NaOH aqueous solution, 4,400ml of 1% DNS (3, 5-dinitrosalicylic acid) solution and 1,275g Rochelle salt were added in an Erlenmeyer flask. One hundred and ten mililiters of 10% NaOH solution, 45g of crystalline phenol and 340ml of distilled water were mixed in another flask, and filled up to 500ml with water. Forty five grams of $NaHCO_3$ and the DNS solution prepared above were added sequentially to this solution.

The mixture was stirred to dissolve Rochelle salt completely, and was filtered after two days. About 6,000ml of DNS reagent was prepared. Three mililiters of DNS reagent was added to 1.0ml of sample solution (0.2-0.2mg glucose) in a test tube. The tube was heated in boiling water for 5min, and cooled quickly under cold running water, to which 16ml of water was added and mixed thoroughly. The optical density of the solution at 550nm was measured.

3. Preparation of protoplasts.

1) Preparation of mycelial homogenates.

Five or six sclerotia from the stock culture were inoculated to 100ml of seed culture medium in a Sakaguchi flask. The flask was stood still at 30°C overnight and shaked reciprocally for 4 days at this temperature. Young mycelia were collected by centrifugation, washed twice with sterilized water, and homogenized with a small amount of water by warring blender for 20sec. The concentration of mycelial homogenates was so adjusted for the absorbance of the solution to be 12.5 O.D. (at 610nm) with sterilized water.

2) Isolation and purification of protoplasts.

Protoplasts were prepared by enzymatical digestion of cell wall. We used two commercial cellulases, chitinase-GODO and β-glucuronidase. The pH of the

mixture was adjusted by 0.25M phosphate buffer (pH 6.0).

Mycelial homogenates (0.2ml), mannitol solution (1.14M, 0.7ml), and the enzyme mixture (0.1ml) were mixed in a test tube, and incubated at 30°C for 2 hrs with reciprocal shaking (120 strokes/min). The mixture was filtered through sterilized G2 filter. The residue was washed with 2.0ml of sterilized mannitol solution (0.8M).

Crude protoplasts in the filtrate and the wash were precipitated by centrifugation (2,500rpm, 5min) and suspended in 2.0ml of 0.8M mannitol solution.

The suspension was covered with 4.0ml of 0.9M sucrose solution cautiously so that two layers were formed. On centrifugation (1,500rpm, 5min) the content separated into three layers. The top layer, white turbid, was collected[18)20)] with a micropipette, and centrifuged again (2,500rpm, 5min). The precipitates were resuspended in 1.0ml of 0.8M mannitol solution (purified protoplasts) and then the number of protoplasts in a unit volume of the suspension was calculated with hemocytometer.

3) Mutagenesis and regeneration of protoplasts.

Purified protoplasts suspension (2.0ml) in a small Petri dish (50mm in diameter) was subjected to irradiation of ultraviolet light for 1min. The suspension was poured on to an agar medium (15ml) in a Petri dish (90mm in diameter), followed by addition of 10ml of soft agar medium and agitation with a glass rod to disperse the protoplasts uniformly. Some mycelial colonies appeared after 3-4 days incubation, and then they were transferred to oat meal agar slants. Deep brown sclerotia were formed usually in 7 days incubation at 30°C and they were stored at 4°C.

4) Production of glucoamylase by mutants.

Five or six sclerotia of the mutants were inoculated to 20ml of the assay medium in a 100ml Erlenmeyer flask and incubated at 30°C overnight. The culture was then incubated at 27-28°C with a rotary shaker for 6 days. The supernatants of the culture broth was obtained by centrifugation (3,500rpm, 10min), and used for assay of glucoamylase activity.

Results and discussion

The amylases produced by *C. rolfsii* are characteristic in their acid tolerance and productivity[17]. Takao et al[15][16]. reported that they hydrolyze raw starch efficiently, especially at high temperature (60-65°C) and low pH (4.0-5.0). In the glucose production on commercial scale, a microorganism which produces such enzyme with high activity and stability is indispensable. So we tried to obtain a new strain by mutagenesis of *C. rolfsii*.

C. rolfsii forms sclerotia, but makes neither fruiting body nor spores. So we prepared protoplasts for ultraviolet light treatment. Younger mycelia were better on their regeneration rates[18][19], we collected the mycelia of 3-4 days cultivation.

For the lysis of cell wall of *Basidiomycetes*, cellulase, chitinase and snail gut enzyme were usually used, but in this study we applied a mixture of cellulases, chitinase, and glucuronidase[19]. As an osmotic stabilizer for the protoplasts, 0.8M mannitol solution was found to be best[19]. The optimum concentration of mycelia was 11.2mg dry weight/ml (Table 1), which corresponds to a solution of O.D. 10 (at 610nm). The photomicrograph of the crude protoplasts obtained is shown in Fig. 3. The number of the crude and purified protoplasts and the rate of recovery are shown in Table 2.

Generally 0.1-1.0% survival dose of ultra-violet light is most effective for mutagenesis. However, since the regenerated colony number was too small, we treated protoplasts at the condition of 10% survival. For the regeneration medium of protoplasts, agar plate was frequently used, in which the sucrose solution of 0.4M was used as osmotic stabilizer. Regeneration process of mycelia from protoplasts are shown in Fig. 4 and 5.

Regenerated colonies were transferred to oat meal slants and 216 strains were obtained (Table 3). Selection of strains which produced glucoamylase activity at more than 120% of the parent strain was done by the following three steps, (1) 54 strains were selected from the 216 strains, then (2) 26 strains from the 54 strains (Table 4) and finally (3) 4 strains from the 26 strains (Table 5).

Encircled numbers in these tables show the selected strains.

Table 1. Dry weight of mycelium homogenates

No.	O.D. (610nm)	Volume	Dry weight
1.	10.01	10ml	0.1188g
2.	10.01	10	0.1515
3.	10.05	10	0.0918
4.	10.05	10	0.1001
mean	10.03		0.1124

Table 2. The number of protoplasts

No.	Crude	Purified	Rate of recovery
1.	2.00×10^6/ml	0.92×10^6/ml	46.0%
2.	2.91×10^6	1.28×10^6	44.0
3.	2.50×10^6	1.07×10^6	42.8
4.	4.86×10^6	1.79×10^6	36.8
5.	3.20×10^6	1.47×10^6	45.9
6.	3.70×10^6	1.15×10^6	31.1
7.	3.11×10^6	1.32×10^6	42.4
8.	3.86×10^6	1.21×10^6	31.3
9.	2.89×10^6	1.04×10^6	40.0
mean	3.09×10^6	1.25×10^6	40.0

Table 3. Enzyme activity of the mutants obtained.

No.	GU/ml	Ratio	No.	GU/ml	Ratio	No.	GU/ml	Ratio	No.	GU/ml	Ratio	No.	GU/ml	Ratio	No.	GU/ml	Ratio	No.	GU/ml	Ratio
Parent strain	15.12	100	31	4.88	32	62	15.08	100	(93)	27.18	180	124	13.64	90	155	15.18	100	(186)	24.42	162
1	10.68	71	32	14.68	97	63	13.55	90	94	1.04	7	125	13.81	91	156	13.65	90	187	17.85	118
2	13.00	86	33	10.80	71	64	11.04	73	95	0.76	5	(126)	20.90	138	157	11.08	73	188	16.93	112
3	16.84	111	34	0.96	6	65	1.66	11	96	1.72	11	(127)	32.00	212	158	1.66	11	189	16.78	111
4	7.12	47	35	12.96	86	66	15.71	104	97	11.48	76	128	14.87	98	159	15.72	104	190	16.69	110
5	16.32	108	36	5.88	39	(67)	18.36	121	98	9.22	61	129	16.81	111	(160)	18.33	121	191	13.30	88
6	15.24	101	37	11.40	75	68	14.93	99	99	4.35	29	(130)	22.57	149	161	14.89	99	(192)	19.51	129
7	13.44	89	(38)	19.41	128	69	14.80	98	100	6.08	40	(131)	35.64	236	162	14.80	98	193	17.53	116
(8)	18.84	125	(39)	30.55	202	70	6.25	41	101	2.42	16	132	6.79	45	163	6.20	41	194	18.04	119
9	15.16	100	40	13.52	89	71	5.75	38	102	13.76	91	133	2.48	16	164	5. 74	38	195	16.18	107
10	17.08	113	41	11.16	74	72	7.26	48	103	12.88	85	(134)	22.21	147	165	7.26	48	196	16.93	112
(11)	19.80	131	(42)	18.45	122	73	7.62	50	104	4.47	30	135	5.47	36	166	7.62	50	(197)	23.22	154
(12)	19.24	127	43	17.28	114	74	3.82	25	105	9.13	60	136	13.68	91	167	3.74	25	(198)	38.78	257
(13)	18.20	120	44	16.75	111	75	0.36	2	106	1.53	10	137	16.72	111	168	0.23	2	(199)	36.65	242
14	16.00	106	45	13.91	92	76	0.92	6	(107)	25.10	166	(138)	24.34	161	169	0.92	6	(200)	26.46	175
15	16.16	107	(46)	27.71	183	77	13.00	86	108	3.90	26	(139)	24.70	163	170	16.66	110	201	11.49	76
16	16.32	108	(47)	25.72	170	78	7.11	47	109	5.62	37	140	11.04	73	(171)	18.14	120	202	13.59	90
(17)	18.40	122	(48)	25.91	171	79	4.54	30	110	6.56	43	(141)	26.44	175	(172)	23.14	153	203	17.08	113
18	19.56	105	(49)	22.08	146	80	12.34	82	111	11.99	79	(142)	22.27	147	(173)	49.52	328	204	16.02	106
19	10.96	72	(50)	28.57	189	81	5.13	34	112	2.55	17	143	13.53	90	(174)	35.29	233	205	15.12	100
20	13.84	92	(51)	19.90	132	82	4.90	32	113	7.35	49	144	0.30	2	(175)	18.60	123	206	13.00	86
21	11.56	76	(52)	24.93	165	83	2.27	15	114	5.96	39	145	1.89	13	176	9.68	64	207	13.45	89
22	13.44	89	53	13.60	90	84	2.03	13	115	10.25	68	146	6.96	46	177	2.63	17	208	10.73	71
23	10.32	68	50	13.72	91	85	0.45	3	116	13.91	92	147	13.21	87	(178)	20.39	135	209	15.27	101
24	11.72	78	(55)	20.81	138	86	4.14	27	(117)	27.71	183	148	4.38	29	179	8.81	58	210	16.02	106
25	14.12	93	(56)	32.10	212	87	7.79	52	(118)	25.76	170	149	12.13	80	180	5.59	37	211	13.60	90
26	12.16	80	57	14.86	98	88	4.28	28	(119)	25.84	171	150	10.87	72	181	16.67	110	212	14.81	98
27	12.64	84	58	16.75	111	89	4.21	28	(120)	22.12	146	151	11.10	73	(182)	20.26	134	213	8.01	53
28	15.48	102	(59)	22.56	149	90	15.20	101	(121)	28.63	189	152	7.41	49	183	17.02	113	214	11.49	76
29	13.12	87	(60)	49.28	236	(91)	24.52	162	(122)	20.01	132	153	9.42	62	184	1.12	7	215	9.07	60
30	6.08	40	61	15.27	101	(92)	28.59	189	(123)	24.90	165	154	15.27	101	185	7.72	51	216	15.12	100

Table 4. Enzyme activity of the selected mutants (1).

No.	GU/ml	Ratio	No.	GU/ml	Ratio	No.	GU/ml	Ratio	No.	GU/ml	Ratio
Parent strain	16.68	100	(51)	22.85	137	120	13.74	82	171	6.17	37
(8)	21.52	129	52	5.57	33	121	7.09	43	172	17.01	102
(11)	23.84	143	(55)	21.92	131	122	10.74	64	(173)	41.76	250
12	4.90	29	(56)	40.35	242	123	18.85	113	(174)	29.52	177
13	14.83	89	(59)	29.19	175	126	16.96	102	175	11.68	70
(17)	28.01	168	60	12.66	76	(127)	42.78	257	178	13.85	83
38	4.57	27	67	15.08	90	130	15.18	91	(182)	21.08	126
39	18.66	119	91	17.73	106	131	18.85	113	(186)	24.43	147
42	7.24	43	(92)	23.10	139	(134)	26.39	158	192	4.20	25
(46)	26.75	160	(93)	23.52	141	138	19.23	115	197	18.77	113
47	18.70	112	(107)	20.74	124	(139)	28.17	169	198	7.23	43
48	14.78	89	(117)	23.24	139	(141)	28.86	173	199	14.18	85
(49)	25.35	152	(118)	24.25	145	(142)	23.82	143	(200)	20.35	122
50	13.58	81	(119)	21.43	129	(160)	21.75	130			

Table 5. Enzyme activity of the selected mutants (2).

No.	GU/ml	Ratio	No.	GU/ml	Ratio	No.	GU/ml	Ratio	No.	GU/ml	Ratio
Parent strain	11.20	100	55	7.99	71	118	12.38	111	160	10.11	90
8	13.22	118	(56)	16.90	151	119	13.26	118	(173)	31.88	285
11	2.50	22	59	12.54	112	(127)	17.02	152	(174)	21.55	192
17	11.64	104	92	12.02	107	134	12.68	113	182	11.31	101
46	9.63	86	93	12.94	116	139	6.54	58	186	12.06	108
49	8.78	78	107	9.86	88	141	10.93	98	200	10.01	89
51	10.11	90	117	12.33	110	142	8.18	73			

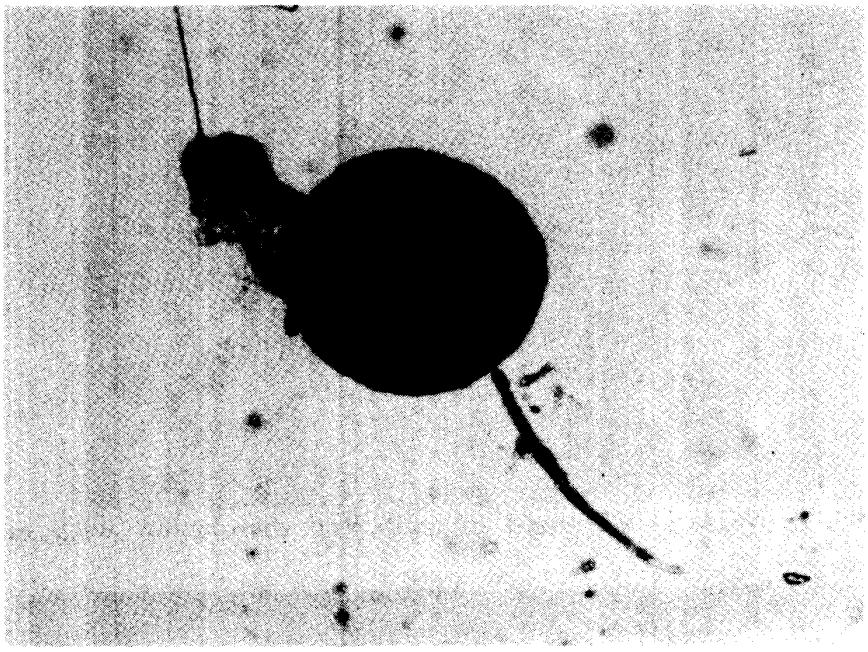

Fig. 1 (×20) Sclerotium

Fig. 2 (×400) Hyphae

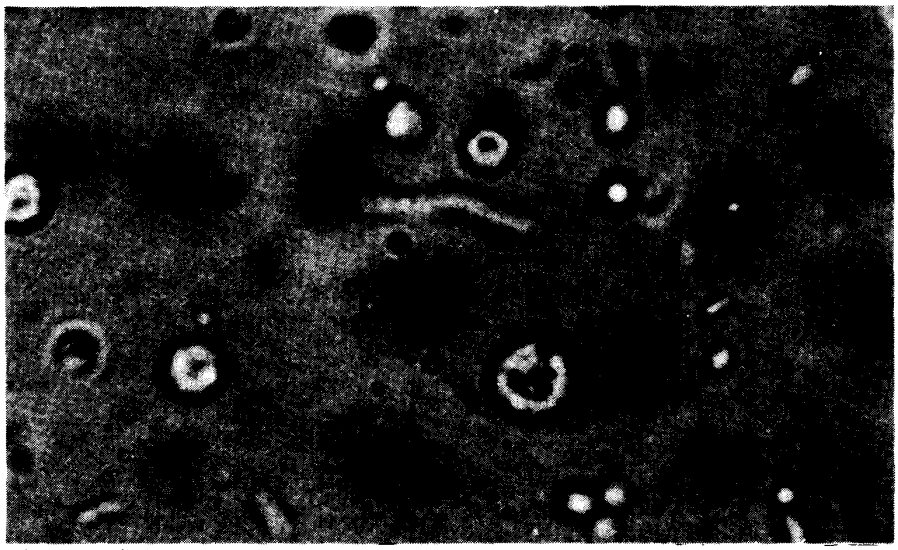

Fig. 3 (×1000) Protoplasts obtained by cell wall dissolution with enzyme mixture.

Fig. 4 (×1000) Regenerated protoplast, 1½ days old.

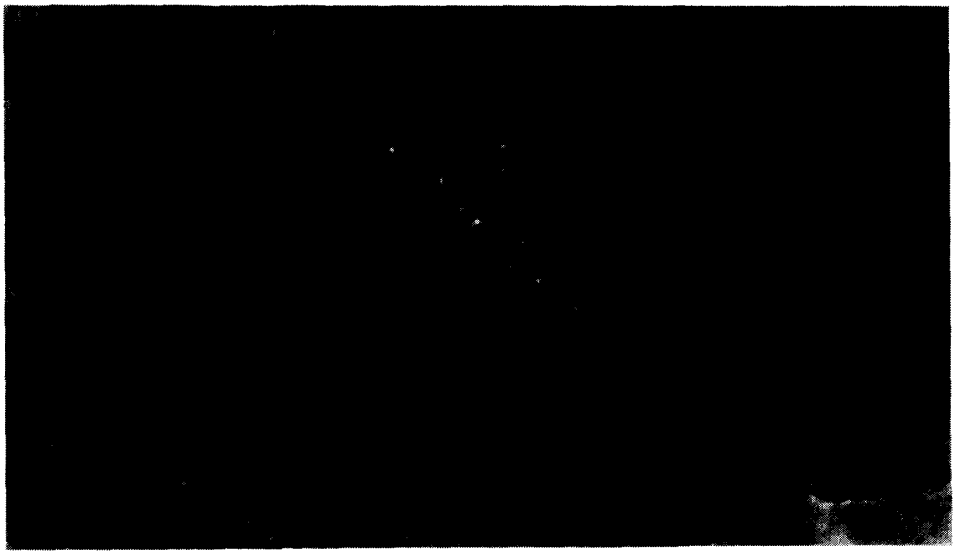

Fig. 5 (×1000) Regenerated protoplast, 2 days old.

Summary

1. The protoplasts of *Corticium rolfsii* were prepared from the homogenates of young mycelia and purified.
2. By irradiation of ultraviolet light on the protoplasts, mutation was induced and the mutants obtained were regenerated on agar plates. They formed sclerotia in a few days.
3. Four strains with higher glucoamylase activity than the parent strain were selected.

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