POLYGALACTURONASE PRODUCTION BY *RHIZOPUS* SPP.

FRANCISCO BANZUELA ELEGADO AND YUSAKU FUJIO*

*Food Science and Technology, Faculty of Agriculture, Kyushu University,
Higashi-ku, Fukuoka 812, Japan*

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Two good polygalacturonase (PG) producing strains were selected from 21 *Rhizopus* spp. isolates from 'ragi' and fermented foods and from 4 authentic *Rhizopus* strains. Strain LKN produced 3.2 U/mg protein of crude enzyme extract (0.68 U/g substrate) in 3 days solid culture of pectin and cassava starch supplemented wheat bran. Strain A11-enzyme showed a wide pH stability range of pH 2–11 and tolerance at 50°C for 20 min.

Pectic enzymes from plant and microbial sources have been studied intensively in the past and they are broadly classified into pectinesterase (PE), which saponifies the esterified portions of the pectin chain, and depolymerases which are responsible for cleaving the chain (17). Among the depolymerases, the major one in terms of hydrolytic function is the endo-polygalacturonase (PG, EC 3.2.1.15). Several review articles that covered the pectic enzymes secreted by various plants and microorganisms, generally reported that plants contain high PE activities. On the other hand, PG is said to be the major pectic enzymes of molds and yeast while bacteria possess higher activities for PE and the lyases (2, 4, 18, 23, 25). But molds seemingly prove to be more useful because of higher pectic enzyme production and optimum activity at a lower pH range suited for the most common industrial use, i.e. juice clarification (3). Hence, a lot of efforts have been devoted to the study of the pectic enzymes by molds. In the case of researches on the pectolytic activity of *Rhizopus* spp., most are related to fruit and vegetable degradation (1). A recent study used citrus waste as substrate for PG production by *Rhizopus oryzae* (7). But in general, it seems that less attention is being paid regarding the utilization of waste substrate material for the culture and screening of *Rhizopus* strains for PG production.

In this study, most of the *Rhizopus* strains used were isolated from 'ragi,' a rice wine inoculum, and some fermented foods, such as 'tempeh' that are native to

* Address reprint requests to: Dr. Yusaku Fujio, Department of Food Science and Technology, Faculty of Agriculture, Kyushu University, 6–10–1 Hakozaki, Higashi-ku, Fukuoka 812, Japan.

Indonesia. This is a more direct method to specifically target the isolation of *Rhizopus* spp., as they are the most commonly found microorganisms in 'ragi' and 'tempeh' (14).

Although problems regarding the reproducibility of data regarding enzyme production in solid cultures may exist (15), solid culture method was employed in this experiment for it is still the preferred method for culturing *Rhizopus* spp. in laboratory researches (13,24) and in industrial applications (19). The genus *Rhizopus* belongs to the family *Mucorales*, that naturally grows in solid medium but hardly thrives in aquatic medium (8).

The major objects of the present work are to obtain a good PG producing *Rhizopus* strain and to decide a culture medium composition for PG production.

MATERIALS AND METHODS

Microorganisms. Five kinds of 'ragi' and many Indonesian fermented foods were used as the isolation sources. Isolation was carried out by plating method on potato dextrose agar (PDA, Nissui Co., Tokyo, Japan) and 2 days incubation at 30 °C. As a result of microscopic inspection of colonies formed on the plate, 21 *Rhizopus* strains were isolated and transferred onto PDA slopes. Four authentic *Rhizopus* strains (*Rhizopus javanicus* IFO 5441 and 5442, and *R. japonicus* IFO 5318 and *Rhizopus* sp. UQM 186F (21)) were used as the reference strains. A total sum of 25 *Rhizopus* strains were used as the PG source strains.

Media and cultivation. Three solid media were used. Medium A consisted of 10 g wheat bran, 0.5 g cassava starch (13,16,26), 1 g pectin (citrus pectin, Ishizu Pharmaceutical Co., Ltd., Osaka, Japan) and 10.5 ml tap water. Medium B excluded cassava starch from medium A and 11 ml tap water was used. Medium C consisted of only 10 g wheat bran and 10 ml tap water. The media were placed in 300 ml Erlenmeyer flask with cotton plug and were autoclaved at 121 °C for 15 min. One milliliter of respective *Rhizopus* spore suspension (ca. 10⁹ spores per ml) was inoculated onto the medium and cultivation was carried out at 30 °C for 4 days, unless otherwise specified. Each strain was cultured in duplicate flasks and the mean of the data from two separate culture runs reported.

Crude enzyme solution. The pectic enzyme was extracted by adding 60 ml of sterile phosphate buffer (K₂HPO₄-KH₂PO₄, 0.05 M, pH 7) to the solid culture in the flask and mixing thoroughly. The mixture was placed in a cold room (4 °C) for 12 h and then the crude enzyme extract was obtained by squeeze and centrifugation. The crude enzyme extract was filtrated with a membrane filter (pore size 0.3 μm, Advantec Co., Ltd., Tokyo, Japan) and the filtrate was dialyzed with visking tube (Sankoh Pharmaceutical Co., Ltd., Tokyo, Japan) in phosphate buffer (0.05 M, pH 7) at 4 °C. The resultant clear liquid was used as the crude enzyme solution after adjusting the same volume for each respective culture.

PG activity assay. The reaction mixture composed of 1 ml of 1% pectic acid (dissolved in acetate buffer, CH₃COONa-CH₃COOH, 0.1 M, pH 4.5) and 0.25 ml of

enzyme solution. The reaction mixture was incubated at 40°C for 30 min unless otherwise specified. One unit of PG activity is defined as the amount of enzyme that releases 1 μ mol of reducing sugar (α ,D-galacturonic acid as a standard) per min at 40°C. The amount of reducing sugar was determined by dinitrosalicylic acid (DNS) method for sugar determination (12).

Temperature and pH dependencies of PG activity. On the basis of PG activity assay, pH optimum of PG was determined at indicated pH by using various buffer pH, i.e., 0.1 M glycine buffer (glycine-HCl, pH 2–3), acetate buffer (pH 3.5–5.5), 0.1 M phosphate buffer (pH 6–8) and 0.1 M sodium carbonate buffer (Na_2CO_3 - NaHCO_3 , pH 8–11). Temperature optimum of PG activity was determined at the range from 20 to 70°C at the optimum pH of the respective enzyme solution. The pH stability of PG was determined by treating the respective enzyme solution at the desired pH (the same buffer as above) for 60 min at 30°C. Heat stability of PG was determined at the respective optimum pH and heating temperature range from 40 to 70°C for heating time range from 5 to 60 min.

RESULTS AND DISCUSSION

PG activities of isolated Rhizopus strains

Table 1 shows PG activities on medium A, B and C for 21 isolates and 4 referred *Rhizopus* strains cultured for 4 days. Based on control culture (without inoculum), A11, LKN, UQM 186F, gave comparatively higher PG activities on medium A. Comparing medium A with medium B and C, medium A proved beneficial in increasing PG activities in almost all strains tested, inducing PG production and promoting mycelial growth (by visual inspection). The supplemented starch and pectin could have been utilized as carbon source for mycelium growth and PG inducer, respectively. The works of Charles and Gavin, as reported by Steinkraus et al. (20), showed that the levels of pectic enzymes in *Rhizopus oligosporus* increased with increase in mycelial growth. However, mycelium growth and PG production in some strains may have been inhibited by pectin or starch supplementation. Among the strains tested, the best 3 PG producers (A11, LKN, and UQM 186F) were selected from the pool of *Rhizopus* strains as first selection step and with IFO 5441 (referred strain) they were subjected to subsequent optimization tests. The PG activities of the selected strains are in the range of 3.3–4.3 U/ml (1.5–2.0 U/mg protein) which are greater than that of reported value of the PG activity of *Rhizopus arrhizus* (0.336 U/mg protein) obtained from rotted apricot (11) and *Rhizopus stolonifer* (1.2 U/ml) grown in synthetic glucose-asparagine medium (9); quite comparable to that of *Aspergillus niger* (1.76 U/mg protein) cultured in a liquid medium containing satsuma mandarin peel (5); but less than that of the reported value of *R. oryzae* (70 U/ml or 29 U/g substrate) grown in orange finisher pulp (7).

Table 1. PG activities of crude enzyme extracts of *Rhizopus* strains cultured for 4 days in three types of solid media.

Strain	PG activity (U/ml) ^a		
	Medium A	Medium B	Medium C
A11	4.3	3.5	3.0
F60	2.3	2.2	1.3
F61	2.1	2.0	1.5
F62	2.5	2.0	0.4
F67	3.2	2.0	2.8
F68	2.1	2.3	1.8
F75	2.6	3.1	1.7
F76	2.0	2.2	0.9
F77	2.2	2.7	1.9
F87	2.3	2.0	1.5
F94	2.5	1.9	1.3
F98	1.6	2.0	1.3
G7	2.5	2.7	1.4
G16	0.8	0.9	0.0
G82	3.1	2.8	1.4
Rh3	2.2	2.7	1.9
LKN	3.9	3.2	2.2
WJ	3.0	2.3	1.5
UM	2.8	2.0	1.6
CJ	2.8	2.3	1.0
ON	2.4	2.6	0.8
UQM 186F	3.3	2.6	2.1
IFO 5318	3.2	2.7	2.3
IFO 5441	2.6	2.3	1.7
IFO 5442	2.1	2.3	2.2

^a All values are means of two trials of solid cultures of the various strains in duplicate flasks.

pH and temperature optima

Figure 1 (a, A11; b, UQM 186F; c, LKN; d, IFO 5441) shows pH optima of 4 enzyme solutions. The enzyme solutions were then subjected to reaction temperatures at their respective optimum pHs. Figure 2 (a, b, c, d) shows temperature optima of the enzyme solution. Optimum pH and temperature of A11, UQM 186F, LKN and IFO 5441 were pH 5 and 55°C, pH 5 and 40°C, pH 4.5 and 50°C, and pH 5.5 and 45°C, respectively. These pH optima are almost similar to the previously reported values for *R. arrhizus* (pH 5) (11) and *R. stolonifer* (pH 4.6–4.8) (22), typical of most fungal PG.

pH and temperature stabilities

Figure 3 (a, A11; b, UQM 186F; c, LKN; d, IFO 5441) shows pH stabilities of the respective enzyme solution. The pH stable range (more than 80% residual activity after 60 min) for A11, UQM 186F, LKN and IFO 5441 were pH 2–11, 4–9, 6–10 and 4.5–8, respectively. Two pH stability peaks were given by UQM 186F strain and IFO 5441 strain, suggesting the probable multiplicity of PG. The pH

Fig. 1. pH optimum of PG produced by selected *Rhizopus*.

a, *Rhizopus* sp. A11; b, *Rhizopus* sp. UQM 186F; c, *Rhizopus* sp. LKN; d, *Rhizopus javanicus* IFO 5441. The buffers used were glycine-HCl (0.1 M, pH 2-3), acetate (0.1 M, pH 3.5-5.5), phosphate (0.1 M, pH 6-8) and carbonate (0.1 M, pH 9-11).

Fig. 2. Temperature optimum of PG produced by selected *Rhizopus*.

a, *Rhizopus* sp. A11; b, *Rhizopus* sp. UQM 186F; c, *Rhizopus* sp. LKN; d, *Rhizopus javanicus* IFO 5441. PG activity was assayed at their respective optimum pH as shown in Fig. 1.

Fig. 3. pH stability of PG produced by selected *Rhizopus*. a, *Rhizopus* sp. A11; b, *Rhizopus* sp. UQM 186F; c, *Rhizopus* sp. LKN; d, *Rhizopus javanicus* IFO 5441. The PG activity was assayed at their respective optimum pH and temperature after incubation for 60 min at desired pH.

stability of strain A11 is remarkably wider when compared to that of *R. arrhizus* (11), *R. stolonifer* (22), and most other fungal PG (2,4) that ranges from 3.5–6.5. Figure 4 (a, b, c, d) also shows temperature stabilities. By heat processing at 40°C for 60 min, all enzyme solutions were stable with residual activities more than 80%. On the contrary, PG activities of all enzyme solutions were almost inactivated by heat processing at 70°C for 5 min. Among the 4 *Rhizopus* strains, A11 showed the best heat tolerance, i.e., stable even by heat processing at 50°C for 20 min. On the other hand, residual PG activities from UQM 186F and LKN were ca. 50% by heat processing at 50°C for 10 min. The PG activity from IFO 5441 shows the weakest heat tolerance. Compared to most fungal PG, the PG of strain A11 possesses higher heat tolerance but is inferior to that of a *Rhizopus nigricans* strain reported by Harper (6).

Optimization of pectin concentration in medium

Figure 5 shows the time courses of PG production by 4 selected *Rhizopus* strains using medium A (see Table 1), which illustrates that every solid culture for 3 days can be sufficient for PG production by selected 4 *Rhizopus* strains. Among the tested strains, the maximum PG production was given as 4.6 PG unit per ml by LKN culture for 3 days and the minimum was that of IFO 5441. On the basis of enzyme productivity, pH and heat stabilities, *Rhizopus* LKN and A11 strains were

Fig. 4. Temperature stability of PG produced by selected *Rhizopus* as a function of heating time.

a, *Rhizopus* sp. A11; b, *Rhizopus* sp. UQM 186F; c, *Rhizopus* sp. LKN; d, *Rhizopus javanicus* IFO 5441. The PG was assayed at their respective optimum pH and temperature.

Fig. 5. Time course of PG production by selected *Rhizopus*.
○, *Rhizopus* sp. LKN; ▲, *Rhizopus* sp. A11; ●, *Rhizopus* sp. UQM 186F; △, *Rhizopus javanicus* IFO 5441. Medium A (Table 1) was used for culturing.

Fig. 6. Effect of increased pectin supplementation on PG production.
●, *Rhizopus* sp. LKN; ○, *Rhizopus* sp. A11. Pectin concentration of Medium A (Table 1) was varied.

Table 2. Comparative PG activities of selected *Rhizopus* isolates and some reported molds.

Strain	PG activity		Culture medium	Reference
	U/ml	U/mg protein		
<i>Rhizopus</i> sp. LKN	8.5	3.2	Wheat bran (solid)	Present work
<i>Rhizopus</i> sp. A11	4.6	1.9	Wheat bran (solid)	Present work
<i>Rhizopus</i> sp. UQM 186F	3.8	1.5	Wheat bran (solid)	Present work
<i>Rhizopus</i> IFO 5441	2.5	1.1	Wheat bran (solid)	Present work
<i>Aspergillus niger</i> K-10	2.7	1.4	Wheat bran (solid)	Present work
<i>Rhizopus arrhizus</i>	2.3	0.4	Apricot (solid)	Liu and Luh, 1978
<i>Rhizopus stolonifer</i>	1.2	—	Glucose-asparagine media (liquid)	Lee and West, 1981
<i>Rhizopus oryzae</i>	70.0	—	Orange finisher pulp (solid)	Hart et al., 1991
<i>Aspergillus niger</i> 35-1	11.7	1.8	Satsuma mandarin (liquid)	Hara et al., 1983
<i>Saccharomyces fragilis</i>	1.6	0.6	Yeast extract medium (liquid)	Lim et al., 1980

Although all PG assays are based on the reducing sugar activity, it must be clear that the PG activity assay and culture methods in references were not exactly the same as the present work. Parenthesis shows solid or liquid culture method.

selected for further optimization. Figure 6 shows PG production for 3 days culture by *Rhizopus* LKN and A11 strains with varying pectin concentration in medium A. Two grams (20% w/w) pectin supplementation produces 8.5 PG units by LKN, but in the case of A11, there is not much effect on PG production by higher pectin concentration. Table 2 shows PG production for 3 days culture on the solid bran medium containing 5% (w/w) cassava starch and 20% (w/w) pectin (medium A supplemented by more pectin) by the 4 selected *Rhizopus* strains, in comparison to four reported microorganisms, wherein the PG activity assay methods done were somewhat similar to our method. Comparing with *Rhizopus* strains tested and some

previously reported microorganisms, it seems that *Rhizopus* sp. LKN could be a good PG producer. It also showed a high degree of pectic acid hydrolysis, cleaving about 12% of the bonds when the viscosity was reduced into half and hydrolyzing about 45% of the bonds after 2 h (data not shown but will appear elsewhere), comparable to that of *R. arrhizus* (11) and *Saccharomyces fragilis* (10). In terms of good pH stability and higher temperature tolerance, *Rhizopus* sp. A11, may also be chosen for further considerations.

CONCLUSION

Among 25 *Rhizopus* strains, *Rhizopus* spp. LKN and A11 isolated from a kind of 'ragi' showed to be promising strains for PG production on wheat bran medium. PG production by strain LKN and A11 were 8.5 and 4.6 PG units per ml of extracted enzyme solution, respectively, on 3-day wheat bran culture, comparable to previously reported *Rhizopus* spp. and other fungal strains. In terms of PG properties of the crude enzyme solution, i.e., pH and temperature tolerance, *Rhizopus* sp. A11 seemed interesting.

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