KOR. JOUR. MICROBIOL. Vol. 22, No. 4, 215~222, 1984

Isolation of *Aspergillus fumigatus* and Properties of It's Enzyme for *Rhodotorula glutinis* Cell Wall Lysis

Pan, Jae-Gu and Joon-Shick Rhee
Department of Biological Science and Engineering, Korea Advanced Institute of Science and Technology, Seoul 131, Korea
(Received November 30, 1984)

Rhodotorula glutinis 세포벽 용해효소를 생산하는 *Aspergillus fumigatus*의 분리와 그 효소의 특성

반 재 구·이 준 식
한국과학기술원 생물공학과
(1984년 11월 30일 수리)

ABSTRACT

A fungus producing cell wall lytic enzyme for *Rhodotorula glutinis* was isolated from local soil and identified partially as a species of *Aspergillus fumigatus* group. The cell wall lytic enzyme was an inducible exoenzyme and composed of at least lytic polysaccharidase and protease which act cooperatively in the lysis of intact cells. The lytic polysaccharidase was not able to hydrolyze β-1,3 and β-1,6-glucan which have the same types of bond as found in the cell wall of *Ascomycetous* yeasts. The lytic polysaccharidase alone was sufficient to hydrolyze the fractionated cell wall (alkali-insoluble residues) of *R. glutinis*, whereas it showed low activity against intact cells.

INTRODUCTION

Enzymes capable of degrading the cell wall of yeasts have been isolated from the culture broth of numerous microorganisms (Phaff, 1977). In general, the yeast lytic activity can be attributed to a β-1,3-glucanase, and its action on intact cells is accelerated by various enzymes such as protease (Scott & Schekman, 1980), phosphomannanase (McLellan *et al.*, 1970), β-1,6-glucanase, and chitinase (Phaff, 1977). The studies on the enzymatic hydrolysis of yeast cell walls were largely confined to the *Ascomycetous* yeasts, whereas there were a few reports on the enzymatic hydrolysis of *Basidiomycetous* yeast cell walls (Lee *et al.*, 1979; Murao *et al.*, 1976; 1978; Phaff, 1977).

We have isolated and partially identified a microorganism producing cell wall lytic enzyme for intact *Rhodotorulas glutinis*, and studied the lytic enzyme composed of lytic polysaccharidase and protease. We believe that this lytic enzyme would be of value in elucidation of the structure of *Rhodotorula* cell wall (Farkas, 1979), formation of spheroplasts from intact cells (Peberdy, 1980), and in mild recovery of intracellular materials or organelles from cells (Asenjo & Dunhill, 1981; Johnson *et al.*, 1979; Knorr *et al.*, 1979; Uzuka *et al.*, 1975).

MATERIALS and METHODS

Cultivation of yeasts

Rhodotorula glutinis NRRL Y-1091 was grown on the medium (in g/*l*): $CaCl_2 \cdot 6H_2O$, 0.2; Na_2HPO_4, 2.0; KH_2PO_4, 7.0; $MgSO_4 \cdot 7H_2O$, 1.5; $(NH_4)_2SO_4$, 1.0; and yeast extract (Difco Lab.), 1.0; the pH was adjusted to 5.5 with NaOH during the cultivation. Glucose (30 g/*l*, technical grade, Miwon Co. Korea) was sterilized separately and mixed aseptically.

Cells were grown in a 10 *l*-Microferm fermentor (M-19, New Brunswick Scientific Co.) for 56 hr, starting with a 5% inoculum of *R. glutinis*. This fermentor was operated at 30°C with aeration of 1vvm at 500 rpm.

Preparation of autolyzed-washed-yeasts

After 56 hr cultivation in a 10*l*-fermentor, cells were harvested by continuous centrifuge (De Laval, 12,000 rpm) at room temperature. The cells were washed twice with distilled water by centrifugation (Sorvall RC-5, Du Pont) at 8,000×g for 20 min at 4°C. Washed cells were suspended in four volumes of distilled water and agitated for 48 hr at 60°C. The insoluble residue collected by centrifugation at 16,000×g for 20 min at 4°C was suspended in an equal volume of distilled water and sonicated with macrotip (80% relative output, Sonic-300 Dismembrator, Artek System Co.) for 10min. The cell suspension was centrifuged at 16,000×g for 15 min at 4°C, and the precipitates obtained from the centrifugation were washed three times with distilled water at the same condition. After extraction of lipid from the precipitates with petroleum ether, residual solvent was evaporated by a rotary vacuum evaporator (R110, Brinkmann). Resulting precipitates (autolyzed-washed-yeasts(AWY)) were kept at −20°C until used.

Assay Substrates

For the assay of intact cell lysis, harvested cells were washed twice with 0.1 M-phosphate buffer (pH 6.0) and stored at 4°C. The cells were used within a week.

For the preparation of alkali-insoluble-residues (AIR) from *R. glutinis* cells in fattening phase (56 hr), a modified method of Manners *et al.* (1974) was used. The cells harvested by continuous centrifugation were washed twice with distilled water by centrifugation at 8,000×g for 20 min at 4°C. Approximately 100 g wet cells were suspended in 1 *l* of 3% NaOH solution, agitated for 9 hr at 60°C, and diluted with an equal volume of distilled water. The suspension was centrifuged at 10,000×g for 30 min at 17°C and the precipitates were resuspended in 500 ml of 3% NaOH solution and agitated for 24 hr at room temperature. Then, the AIR were collected by centrifugation at 16,000×g for 20 min at 17°C, and were washed three times with distilled water at the same condition. After extraction of fat soluble materials from the AIR with petroleum ether, the AIR were freezedried and stored at 4°C until used.

For the assay of lytic polysaccharidase, the freeze-dried AIR was suspended in assay buffer or distilled water to the appropriate concentration; the AIR was finely dispersed by ejecting repeatedly the suspension through syringe.

Other carbohydrate polymers were obtained from various sources and used without further purification: laminarin (ex. *L. digitata*) and pustulan (ex. *P. papullosa*) from Calbiochem, U.S.A., yeast mannan from Tokyo Kasei, Japan, cellulose (Avicel) from Merck, West Germany and soluble starch from Wako, Japan.

Enzyme assays

Lytic polysaccharidase activity was measured at 30°C. The reaction was started by

adding 0.2 ml enzyme solution to an equal volume of prewarmed substrate (AIR, 2 mg/ml in 0.1 M-phosphate buffer, pH 6.0), incubated for 0.5 hr with gentle shaking, and stopped by adding 0.8 ml Nelson's copper reagent. 0.4 ml distilled water was added to the inactivated mixture which was thoroughly mixed, and the reducing sugar concention was deterimined by Nelson's method (Ashwell, 1957). Assays were done in duplicate, with a reaction mixture containing no enzyme as a control. Reducing power of culture broth was determined and calibrated for the measurement of lytic polysaccharidase activity, whenever necessary. One unit of lytic polysaccharidase was defined as the amount of enzyme which liberated 1 μmole of reducing sugars per 0.5 hr at 30°C and at pH 6.0.

Standard assay mixture of protease was composed of 0.2 ml enzyme solution, 0.4 ml 0.1 M Tris-HCl buffer (pH 7.5) and 0.4 ml of azocasein (Sigma Chemical Co., 0.1% solution filtered through Whatman #5 filter paper). All solutions except enzyme were prewarmed to the reaction temperature at 30°C. After 0.5 hr incubation, reaction was stopped by the addition of 10% TCA solution. After standing for 20 min at room temperature, precipitate was removed by centrifugation at 14,000×g for 15 min at 4°C. A_{370} of supernatant solution was measured against a blank containing no enzyme. One unit of protease was defined as the amount of enzyme needed to produce an increase in one A_{370} unit per 0.5 hr under the standard assay condition.

Molecular exclusion with Bio-Gel P-60

After 30 hr culture on induction medium, culture fluid of the fungus was filtered through Toyo #2 filter paper and centrifuged to discard insoluble materials at 12,000×g for 20 min at 4°C. Then, it was concentrated to about one-tenth of its original volume by ultrafiltration using UM-2 membrane (Amicon Corp.). The concentrated-culture fluid was dialyzed against 10 mM-sodium succinate buffer (pH 6.0) for 24 hr at 4°C with the exchange of buffer every 6hr. The dialyzed-concentrated-culture fluid was ready for the column chromatography.

Molecular exclusion was done with Bio-Gel P-60 (Bio-Rad Lab.). Column (54×2.5 cm; 265 ml bed volume; 78 ml void volume) was equilibrated with 50 mM-sodium succinate buffer (pH 7.0), and eluted with the same buffer by gravity flow. Fractions of 4.8 ml were collected at 21.6 ml/hr.

RESULTS

Isolation and partial identification of cell wall lytic microorganism

Enrichment culture was set up by inoculating suspensions of various soil samples on the double layer agar plate (1.0% AWY, 1.0% K_2HPO_4, 0.01% $MgSO_4 \cdot 7H_2O$, and 2% agar in upper layer; 1.0% K_2HPO_4, 0.01% $MgSO_4 \cdot 7H_2O$, and 1% agar in lower layer). AWY cells served as a source for both nitrogen and carbon. Relatively high pH of the screening medium (pH 7.6) suppressed the normally occurring abundant mycelial growth, resulting in the simplification of microbial flora, and thus facilitating the isolation of lytic microorganisms. The development of a double layer agar plate technique provided the best way of distinguishing halos produced by colonies of yeast cell wall lytic microorganisms. Halos appeared after several days' incubation at 30°C. Colonies of *R. glutinis* cell wall lytic microorganisms were purified by the repeated transfer to the above screening medium. Eight isolates included seven strains of molds and mold-like microorganisms and one strain of bacterium.

Based on the selection criteria listed in

Table 1. Criteria for the selection of cell wall lytic microorganisms

* Degree of clearance of halo made by lytic microorganisms.
* Growth rate in submerged culture and plate culture.
* Activity on the intact cells of *R. glutinis* in fattening phase-using the assay method described in Fig. 1.
* Microscopic observation for the spheroplast formation.

Table 1, the lytic fungus was selected for further research. The lytic fungus was identified as a species of *Aspergillus fumigatus* group. The observed characteristics of the fungus were reported in detail by Pan (1982).

Conditions for the synthesis of lytic enzyme

Results of the exploratory experiments showed that the lytic enzyme was an inducible exoenzyme, and conditions for the lytic enzyme synthesis were as follows: Induction medium contained 1% AWY cells, 0.5% malt extract and salt components taken from Czapek-Dox agar media; the malt extract was incorporated to stimulate initial growth of the fungus; initial pH of induction medium was adjusted to 5.6 (optimal between pH 5.0 and 6.5). The fungus was cultured in 250 ml Erlenmeyer flasks which contained 50 ml of induction medium (150 rpm, 30°C).

Lysis of intact cells by crude lytic enzyme

The *R. glutinis* cell wall-induced lytic enzyme preparation was tested for its ability to produce spheroplasts of *R. glutinis*. When this lytic enzyme preparation was incubated with intact cells for 6 hr, the spheroplasts were clearly visible under light microscope (1,000×).

Intact cells in growing phase were more susceptible to lysis than those in fattening phase as shown in Fig. 1. However, degree of cell wall removal was observed not to be differentiating between spheroplasts of growing cells and those of fattening cells.

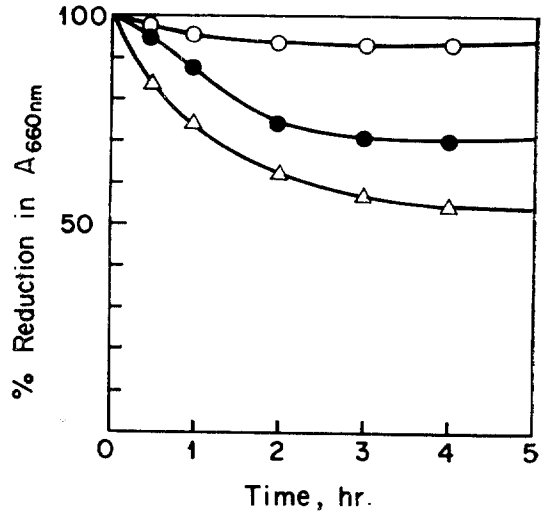

Fig. 1. Lysis of intact cells in growing phase and fattening phase.
Reaction mixture (A_{660}nm, 1.0~1.1) were composed of 5.0 ml of washed yeast cell suspension in lytic enzyme preparation. Reactions were incubated with gentle shaking at 30°C, (○----○) control (no enzyme added), (△----△) cells in growing phase (30 hr), (●----●) cells in fattening phase(56 hr). 0.01% sodium azide was used as an antimicrobial preservative. Control run to follow yeast autolysis used cells in fatttening phase.

Action of crude lytic enzyme preparation on various polysaccharides

The culture fluid of the fungus grown on the induction medium was filtered through Toyo #2 filter paper, centrifuged at 16,500 ×g for 20 min at 4°C, and tested for its polysaccharide hydrolyzing activities on the substrates listed in Table 2. The culture fluid of the fungus showed relatively low level of β-1,3-and β-1,6-glucanase activities in comparison with those of *Bacillus circulans* WL-12 (Tanaka & Phaff, 1963), *Oerskovia xanthioneolytica* (Scott & Schekman, 1980). and *Paecilomyces lilacinus* ATCC 36010 (Arai & Murao, 1978).

β-1,3-Glucanase, which hydrolyzes β-1,3-

Table 2. Action pattern of crude lytic enzyme preparation on various polysaccharides

Substrate	Predominant types of bonds	Hydrolysis
Cellulose	β-1,4-	−
Starch	β-1,4-, β-1,6-	−
Yeast mannan	β-1,3-, β-1,2-, β-1,6-mannose	−
Laminarin	β-1,3-	+
Pustulan	β-1,6-	slight
AWY	Glucomannan(?)	+
AIR	Glucomannan β-1,4-, β-1,3-(?)	+

Reaction mixtures contained 0.5 ml phosphate buffer (pH 6.0, 0.1 M), 0.5 ml crude enzyme preparation and 1 ml substrate (1 mg/ml). Reactions were proceeded for 3 hr at 30°C. Individual control runs which were stopped at time zero were made for the measurement of reducing sugars in the orginal reaction mixtures.

glucan normally normally found in the cell walls of *Ascomycetous* yeasts, was also found in the culture broth of the fungus. However, it was uncertain whether β-1,3-glucanase acts in *R. glutinis* cell lysis. Hence, fractionation of the crude lytic enzyme was necessary to clarify this question.

Assay of lytic polysaccharidase

Assay of lytic polysaccharidase with intact cells or AWY cells was unsatisfactory for the measurement of enzyme quantity due to its insensitivity. AIR of *R. glutinis* cells in their fattening phase were prepared according to the scheme of cell wall fractionation proposed by Manners *et al.* (1974). Manners *et al.* insisted that AIR of yeasts have generally a very heterogeneous structure, which is responsible for the rigidity of yeast cell walls.

Assay of lytic polysaccharidase with AIR turned out to be relatively sensitive, although composition of AIR of *R. glutinis* was not determined. Perhaps, AIR of *R. glutinis* would be composed of glucomannan (Arai & Murao, 1978; Phaff, 1977). Thus, routine assay of lytic polysaccharidase was done by measuring the linear rate of reducing sugar production at 30°C with 2% AIR in 0.1 M-phosphate buffer (pH 6.0).

Fractionation of the lytic enzyme

In many cases, the cell wall-induced lytic enzyme, which is capable of promoting living

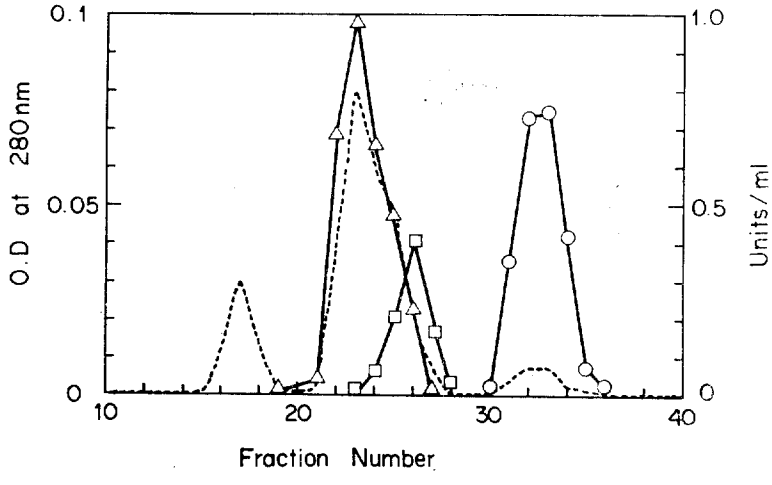

Fig. 2. Bio-Gel P-60 column chromatography.
Column (54×2.5 cm; 265 ml, bed vol.; 75 ml void vol.) the assay of β-1, 3-glucanase was done with the procedure same as that of the lytic polysacharidase, (--------) O.D. at 280 nm, (△----△) lytic polysaccharidase, (□-----) β-1, 3-glucanase, (○----○) protease.

cell lysis, is known to consist of complex enzymes such as phosphomannanase (McLellan *et al.*, 1970), protease (Scott & Schekman, 1980) and other glucanases (Phaff, 1977). Thus, fractionation of lytic enzyme was tried.

The dialyzed-concentrated-culture fluid prepared by the procedure described in experimental section was applied to Bio-Gel P-60 column. Assay of protease was tried because of frequent reports on the protease cooperativity in living cell lysis. As shown in Bio-Gel P-60 chromatogram (Fig. 2), at least three enzyme activities were present.

Lytic polysaccharidase activity was recovered at the second protein peak and was contaminated somewhat with β-1, 3-glucanase activity. However, fractions (#22 and 23) were not contaminated with β-1, 3-glucanase activity, and were judged to have uniform specific activity. That is to say, lytic enzyme could be separated from β-1, 3-glucanase, not by protein peak, but by activity peak. Protease activity appeared in fractions (#31-34), was pooled, and used for further experiments.

Fractionated lytic polysaccharidase was not able to hydrolyze the laminarin and pustulan which were known to have the same type of bonds as found in the cell walls of *Ascomycetous* yeasts.

Cooperativity of lytic polysaccharidase with protease

Fractionated lytic polysaccharidase had no activity against intact cells of *R. glutinis*, whereas it has hydrolytic activity on the fractionated cell wall (AIR) of *R. glutinis*. This result suggested that another component from culture broth acts together in living cell lysis. For the effective lysis against intact cells, as least lytic polysaccharidase and protease were required (Fig. 3). Spheroplasts formation was also observed in the reaction mixture containing reconstituted lytic polysaccharidase plus protease.

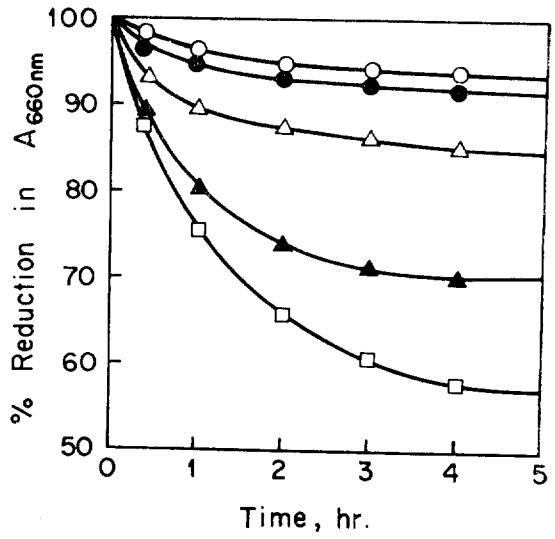

Fig. 3. Lytic polysaccharidase and protease that act together in the lysis of intact cells. Reaction mixtures contained 1 unit of lytic polysaccharidase and 0.45 unit of protease in 5 ml suspension of intact cells (50 hr). 0.01% sodium azide was used as an antimicrobial preservative. (○----○) control, (△----△) protease, (●----●) lytic polysaccharidase (▲----▲) protease + lytic polysaccharidase (□----□) original culture broth.

DISCUSSION

With the purpose of mild recovery of intracellular materials and organelles from yeasts, a fungus producing cell wall lytic enzyme for *R. glutinis* was isolated from soil. The fungus was identified as a species of *Aspergillus fumigatus* group. Some isolated strains producing these types of enzymes were reported to be identified as the strains of *Paecilomyces lilacinus* (Murao *et al.*, 1976) and *Bacillus pumilus* (Murao, *et al.*, 1978; Lee *et al.*, 1979). Thus, we believe that our finding in this study is the first one reporting that a species of *Aspergillus fumigatus* can produce cell wall lytic enzyme for *Rhodotorula* yeasts.

Lytic enzyme of the fungus was able to lyse the intact as well as fractionated cell walls (AIR) of *R. glutinis*. Lytic enzyme was, at least, composed of two components; lytic polysaccharidase and protease. For the lysis of intact cells, lytic polysaccharidase and protease must act cooperatively. However, lytic polysaccharidase alone was sufficient to hydrolyze the fractionated cell walls (AIR) of *R. glutinis*. This result well supports the hypothetical structure of yeast cell wall, which suggests the mannoprotein complexes be present on the outer layer of glucan (Farkas, 1979). That is to say, the glucan layer of cell wall, which is responsible for structural rigidity and integrity, is covered by a layer of mannoprotein which must be modified in some way, before a lytic polysaccharidase can reach its substrate and promote cell lysis. In view of this conjecture, cell wall of *R. glutinis* may be judged to have a general structure similar to other yeasts.

However, the glucomannan (?) layer of *R. glutinis* cell wall has different structure from that of *Ascomycetous* yeasts (Arai & Murao, 1978; Phaff, 1977). This fact again was supported by our result that lytic polysaccharidase of the fungus was not able to hydrolyze the laminarin and pustulan, which have the same bonds as found in the glucan layer of *Ascomycetous* yeasts.

At this point, it is reasonable to think that this lytic polysaccharidase of the fungus could be used as a tool for elucidation of the cell wall structure of *R. glutinis* (*Basidiomycetous* yeasts), although specificity of lytic polysaccharidase of the fungus remain obscure.

Treatment of intact *R. glutinis* cells with lytic enzyme results in the formation of spheroplasts, which could be exploited as basic tools in the research with *Rhodotorula* yeast, such as yeast transformation and physiological study (Peberdy, 1980).

Enzymatic hydrolysis of *R. glutinis* cell walls provides a way of isolation of the intracellular globule containing storage fats (Uzuka *et al.*, 1975). Accordingly, use of wall-lytic enzymes should help us to understand the nature of storage lipid of oleaginous yeasts, a potential source of microbial fat production.

ACKNOWLEDGEMENTS

This work was supported in part by Korea Science and Engineering Foundation.

적 요

Rhodotorula glutinis 세포벽에 작용하는 용해효소 생산곰팡이를 토양으로부터 분리하였고, *Aspergillus fumigatus*에 속하는 species로 동정되었다. 이 세포벽 용해효소는 세포외 유도효소였으며 lytic polysaccharidase와 protease로 구성되어 생세포 용해에 공동으로 작용하였다. 이 lytic polysaccharidase는 *Ascomycetous* 효모에서의 주 구성 결합인 β-1,3- 와 β-1,6-glucan에는 작용치 않았다. 이 효소는 생세포에는 역가가 낮았지만 *R. glutinis*의 분획된 세포벽에는 protease의 도움없이 작용할 수 있었다.

REFERENCES

Arai, M. and S. Murao, 1978. Characterization of oligosaccharides from an enzymatic hydrolysate of red yeast cell walls by lytic enzyme. *Agr. Biol. Chem.*, **42**:1651-1659.

Asenjo, J.A. and P. Dunhill, 1981. The isolation of lytic enzymes from *Cytophaga* and their application to the rupture of yeast cells. *Biotechnol. Bioeng.*, **23**:1045-1056.

Ashwell, G., 1957. Colorimetric analysis of sugars. p. 85-96. In S.P. Colowick and N.O. Kaplan(ed), *Methods* in *Enzymology, Vol. 3, Academic Press*

Inc., NY.

Farkas, V., 1979. Biosynthesis of cell walls of fungi. *Microbiol. Rev.*, **43**: 117-144.

Johnson, E.A., T.G. Villa, M.J. Lecois, and H.J. Phaff, 1979. Lysis of the cell wall of the yeast *Phaffia rhodozyma* by a lytic enzyme complex from *Bacillus circulans* WL-12. *J. Appl. Biochem.*, 1:273-282.

Knorr, D., K.J. Shetty, and J.E. Kinsella, 1979. Enzymatic lysis of yeast cell walls. *Biotechnol. Bioeng.* **21**:2011-2021.

Lee, T.H., M. Arai, and S. Murao, 1979. Some properties of lytic enzyme for red yeasts prepared from *Bacillus pumilus*, *J. Ferment. Technol.* 57:32-38.

Manners, D.J., A.J. Masson, and J.C. Patterson, 1974. The heterogeneity of glucan preparations from the walls of various yeasts. *J. Gen. Microbial.* **80**:411-417.

McLellan, W.L. JR., L.E. McDaniel, and J.O. Lampen, 1970. Purification of phosphomannanase and its action on the yeast cell wall. *J. Bacteriol.* **102**:261-270.

Murao, S., T.H. Lee and M. Arai, 1978. Identification of a bacterium which produced cell wall lytic enzyme for red yeast and purification of the enzyme. *J. Ferment. Technol.* **56**:472-476.

Murao, S.,R. Yamamoto, and M. Arai, 1976. Isolation and identification of red yeast cell wall lytic enzyme producing microorganisms. *Agr. Biol. Chem.* **40**:23-26.

Pan, J.G., 1982. Enzymatic degradation of *Rhodotorula gracilis* cell wall. *M.S. Thesis*, KAIST.

Peberdy, J.F., 1980. Protoplast fusion-a tool for genetic manipulation and breeding in industrial microorganisms. *Enzyme. Microb. Technol.* **2**:23.

Phaff, H.J. 1977. Enzymatic yeast cell wall degradation. *Adv. Chem. Ser.* **160**:244-282.

Scott, J.H., and R. Schekman, 1980. Lyticase: endoglucanase and protease activities that act together in yeast cell lysis. *J. Bacteriol.* **142**: 414-423.

Tanaka, H. and H.J. Phaff, 1963. Enzymatic hydrolysis of yeast cell walls: I. Isolation of wall-decomposing organisms and separation and purification lytic enzymes. *J. Bacteriol.* **89**:1570-1580.

Uzuka, Y., T. Kanamori, T. Koga, and T. Nagamura, 1975. Isolation and chemical composion of intracellular oil gloubles from the yeast *Lipomyces starkeyi*. *J. Gen. Appl. Microbiol.* **21**: 157-168.