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Production of Pectic Enzymes and Its Properties for Citrus Wine Making

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柑橘醱酵酒의 淸澄化를 위한 펙틴分解酵素의 生産과 그 酵素의 特性

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Summary

In order to produce clear and favorable citrus wine, isolation of useful strain capable of hydrolase-producing, production of enzyme and its properties, application of enzyme to wine making were investigated. Enzyme activities of twenty-one strains of *Aspergillus* sp. isolated from field soils of Cheju island, and four strains preserved in our laboratory were determined. Among these, the strain CCM-4 was selected as a best strain for this purpose, and was identified as *Aspergillus niger* CCM-4. Optimum conditions of this strain for the production of pectic enzyme were for 3 days cultivation at 30°C on solid culture of wheat bran or mandarin orange peel. Optimum conditions for enzyme reaction were pH 4.0 and 40°C, respectively. Enzyme treated wine was cleared much sooner and could be filtered much more rapidly than the untreated.

INTRODUCTION

About 6×10^4 tons of citrus fruit was consumed per year for canning and juice manufacturing processes among 3.7×10^5 tons produced in Cheju island in 1985, and production of citrus fruits will be increased annually. Development of new citrus processing products other than juice or canning will also be significant in consuming

citrus fruits efficiently. Wine making would be one of the useful resolutions among these.

It is desirable that new wines be made clear and free of sediment as soon as possible after fermentation. Brilliantly clear wines are not only more attractive in appearance than the cloudy or hazy, but also, because early removal of suspended yeast cells and sediment forestalls certain undesirable changes in flavor, bouquet and stability, they are of higher quality (Cruess et al. 1955). However, brilliantly clear wine would not be made easily because of pectic substances containing in citrus fruits, and sediment would be caused very slowly according to a long lapse of time. One means of hastening clearing is by the use of pectic enzymes. They have been investigated for many years in the clarification of apple juice or orange juice(Fukui and Nomura 1971, Lee 1977, Lee and Chang 1971). However, there are some reports on the application of pectic enzymes to wine making from apple or grape(Cruess et al. 1955, Lee 1977, Lee and Chang 1971, Raper and Fennell 1973, Reed 1975), no reports was found to mandarin orange wine making for clarification.

It is well known that many microorganisms produce several pectic enzymes, but only a few reports were published on pectic enzymes applied to wining recently(Lee 1977, Reed 1975). In this work, we isolated strains from field soils in Cheju island, which could produce hydrolyzing enzymes. Production of pectic enzymes and its characteristics, and its application to citrus wine making were investigated.

MATERIALS AND METHODS

Microorganisms

Twenty-five strains of fungi of *Aspergillus* sp. preserved in the Biochemical Engineering Laboratory of Cheju National University, and isolated from field soils in Cheju island were examined for hydrolyzing enzymes activities. Among these, the strain which was identified as *Aspergillus niger* CCM-4 is used throughout this work. The stock culture was maintained on slant culture of YM-medium.

In fermentation of mandarin orange juice, *Saccharomyces cerevisiae* preserved in author's laboratory was used.

Midium and culture conditions

Isolation medium of strains capable of producing hydrolase is shown in Table 1. Pectin or CMC was used as a carbon source, and the strains were isolated by means of plate cultures. Fungi to be tested in the determination of enzymes activities were inoculated to a culture medium containing wheat bran or orange peel in a 500 ml flask, and cultured for 3 days at 30°C.

Table 1. Isolation medium of hydrolase-producing strains

CMC or Pectin	5.0 g
$NaNO_3$	2.0
K_2HPO_4	1.0
$MgSO_4 \cdot 7H_2O$	0.5
KCl	0.5
$FeSO_4 \cdot 7H_2O$	0.01
Agar	20.0
Deionized water	1.0 ℓ
pH	5.0

Taxonomy

All the tests used in the taxonomic studies were carried out according to "The Genus *Aspergillus*" (Raper and Fennell 1973).

Assay of enzyme activity

After cultivation, deionized water was added to the flask, extracted and centrifuged(3,000 rpm,

15 min). The supernatant was used as a crude enzyme. Hydrolytic activity of polygalacturonase, xylanase, amlyase, CMC ase was assayed against citrus pectin, xylan, soluble starch and CMC as substrates, respectively. Enzyme activities were determined by the method of authors (Lee and Koh 1975, Lee et al. 1976), and reducing sugar was determined by the modified Somogyi-Nelson method (Hatanaka and Kobara 1980).

One unit of enzyme in the screening of the strains is defined as the amount of enzyme that releases 1 μmol of reducing sugar from a given substrate per min at 40°C.

Enzymatic reaction of pectinase was carried out as a substrate of 1 % citrus pectin at 40°C, pectinase activity was determined by viscosity reduction with Ostwald viscometer at 15°C (Lee and Koh 1976) and citrus juice-clarifying activity (Lee and Chang 1971).

Juice-clarifying activity was determined as follows. Crude enzyme solution(0.5 ml)was added to 5 ml of mandarin orange juice in centrifugal tube(1.5×10 cm), and incubated at 40°C for 2 hrs. After centrifugation of reaction mixture (3,000 rpm, 5 min), supernatant was diluted to 5 times, and absorbance was determined at 660 nm. Juice-clarifying activity was expressed as follows.

$$\text{Juice-clarifying activity}(\%) = \frac{\text{Absorbance of Blank} - \text{Absorsance of Sample}}{\text{Absorbance of Blank}} \times 100$$

crude enzyme solution was salted out by ammonium sulfate between 0.2 and 0.8 saturation. After dialysis of the precipitate collected by centrifugation(7,000 rpm, 30 min), reaction properties of enzyme were investigated.

Fermentaion of citrus juice

Citrus wine making was carried out as follows. After extraction of peeled mandarin orange, juice was diluted to two times by addition of tap water or supernatant of cultured medium of *Aspergillus niger* CCM-4 as described in Materials and Methods, and was adjusted to Brix 24 by addition of sugar. *Saccharomyces cerevisiae*, cultivated on orange juice medium for 24 hr at 30°C, was inoculated to the adjusted orange juice, and fermentation was carried out for one month at room temperature.

Analytical methods

After fermentation, alcohol concentration was measured with hydrometer after distillation of fermented orange juice. Methanol content was determined by the method of AOAC(Horwitz ed. 1975). Optical density of the supernatant was measured at 660 nm with PRE Unicam visible spectrophotometer.

RESULTS AND DISCUSSION

Screeing of hydrolase-producing fungi

More than 300 strains were isolated form field soils and plants of 250 samples collected in Cheju island. Among these strains, enzyme activities of 21 isolated fungi and 4 strains preserved in author's laboratory which were supposed to be *Aspergillus* sp. were determined. A total of 25 strains of fungi were screened for the production of hydrolase under the conditions as dedscribed in Materials and Methods.

The ten good producers are listed in Table 2. among them, strain CCM-4 was selected as one of the best producers of hydrolase from the culture medium containing wheat bran and orange peel. Morphological characteristics of the strain CCM-4 is shown in Table 3. After cultivation

Table 2. Comparision of hydrolase—producing ability among several strains of *Aspergillus* sp.

Strain	Enzyme activity(unit/*ml*)			Viscosity reduction(%)	
	CMCase	Xylanase	Amylase	CMCase	Pectinase
CCM 1	0.11	0.09	0.48	38.1	59.1
3	0.11	0.15	0.53	23.4	56.9
4	0.11	0.23	0.55	33.7	58.4
11	0.11	0.19	0.50	30.0	59.4
12	0.09	0.20	0.50	43.0	55.6
14	0.10	0.23	0.51	31.1	57.4
15	0.09	0.20	0.55	35.4	52.6
22	0.10	0.22	0.49	35.8	56.8
M-2093	0.10	0.26	0.53	35.8	53.3
M-2299	0.10	0.24	0.55	30.3	55.9

Table 3. Morphological characteristics of isolate CCM—4

Colony character		
	rate of growth	ordinarily rapid growth
	texture	closely rough velvety
	color above	carbon black
	color reverse	orange brown
Cordinal heads		
	color	dark brown
	form	globose
	size	80–180 μ
Conidiophore		
	color	dark brown
	markings	comparatively smooth
	length	1.5–2.5㎜
	diameter	15–35 μ
Vesicle		
	color	pale brown
	shape	glocose to subglobose
	size	45–50 μ
Sterigmata		
	color	commonly pale brown
	form	biseriate

Primary sterigmata	
length	15–20 μ
width	3.5–4.0 μ
Secondary sterigmata	
length	5.0–6.0 μ
width	2.5–3.0 μ
Conidia	
color	dark brown
shape	globose
size	3.5–4.0 μ

* Czapek's agar medium(pH 5.0) at 30°C, for 5 days incubation.

of this strain on Czapek's medium for 5 days at 30°C, morphological properties of this strain was very similiar to *Aspergillus niger* described in "The Genus *Aspergillus*"(Raper and Fennell 1973). This strain was identified as *Aspergillus niger* CCM–4.

The U. S. Food and Drug Administration has stated that pectinases prepared in accordance with "good manufacturing practiece" as defined by the FDA and derived from *Aspergillus niger* are "generally recognized as safe"(GRAS) in the United States(Reed 1975), and *Aspergillus* sp. has been used in fermented foods such as soybean souce and paste in oriental countries. Athough it is well known that many microorganisms produce several pectic enzymes(Reed 1976), the strains of *Aspergillus* sp. only were screened and investigated in this work.

Production of pectic enzyme

Viscosity reduction is mainly derived from endo–polygalacturonase, and its activity contributes to clarify fruit juice(Lee and Chang 1971). Therefore, enzyme activities were determined by the method of viscosity reduction in this work. Maximum enzyme production of this strain were for 3 days cultivation at 30°C on wheat bran or mandarin orange peel as shown in Figure 1.

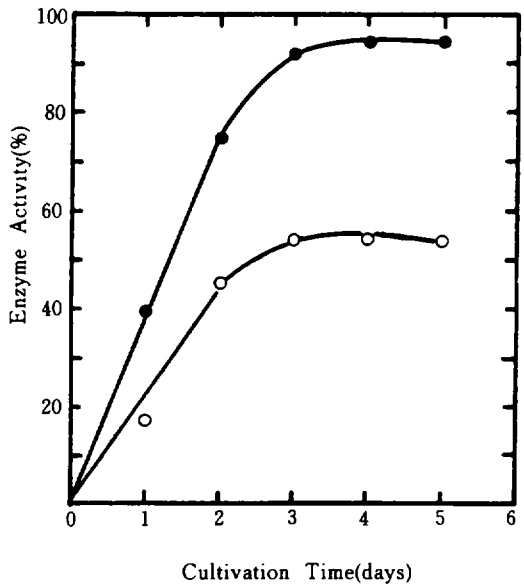

Fig.1. Time–course of pectolytic enzymes formation by *Aspergillus* niger CCM–4 on a wheat bran medium. Viscosity reduction activity(○) and citrus juice–clarifying activity(●) were assayed against 1 % pectin and mandarin orange juice.

There were not any differences of enzyme activities in pH of medium between pH 3.0 and 7.0 This would be derived from pH compensation

between buffer solution and solid medium. Small amount of buffer solution did not so much affect the maintaining pH of solid medium. Various carbon and nitrogen sources were tested for pectic enzyme productions, but not so much differences were found between these. Orange peel seemed to be the place of wheat bran as a carbon source.

Properties of pectic enzyme

Solid ammonium sulfate was added to the culture filtrate between 0.2 and 0.8 saturation. The precipitate formed was collected by centrifugation(7,000 rpm, 30 min), dissolved in a small volume of deionized water, and the solution was dialyzed against deionized water overnight. Properties of pectic enzyme were determined with dialyzed enzyme solution. Optimum conditions for crude enzyme reaction were pH 4.0 and 40°C, respectively, as shown in Fig. 2 and Fig. 3. After crude enzyme was treated at the temperatures between 30°C and 70°C for 1 hr, respectively, enzyme activities were assayed.

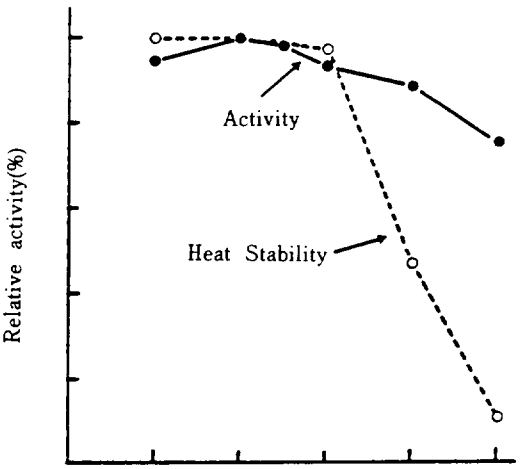

Fig. 2. Effect of temperature and heat stability on the activity of pectic enzyme.

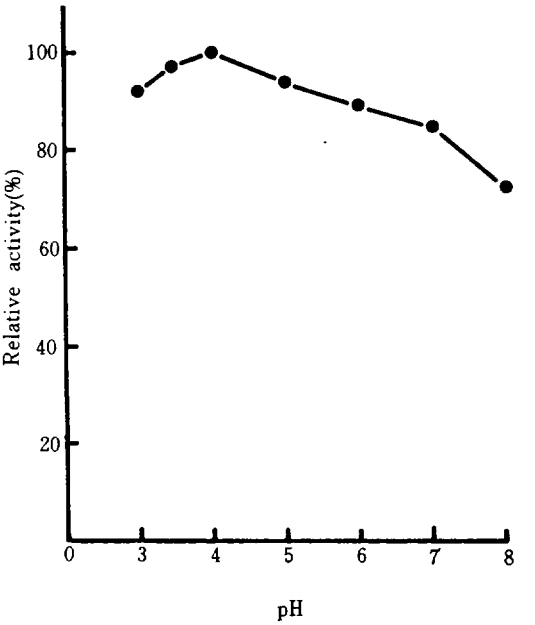

Fig. 3. Effect of pH on the activity of pectic enzyme.

Most of crude enzyme activity was stabilized under 50°C, and about half of activity was remained even at 60°C. The pH of orange juice is between 3.0 and 4.0. Optimum pH for enzyme reaction of this strain seems to be useful for application to clarify in citrus wine making.

Application of pectic enzyme to wine making

It was found that enzyme treated wines were cleared much sooner and could be filtered much more rapidly than the untreated. Fungal crude enzyme preparations contain not only pectic enzymes but also several enzymesCruess et al. 1955). therefore, this enzymes would contribute to spilt cellulose, pentosnas, pectin and other polysaccharides. Citrus wine would be clarified by the action of this enzymes. At 2 weeks the enzyme treated wine was briliiantly clear and the unterated was still cloudy. The untreated-wine was failed to clear in 3 months' aging, and

Table 4. Chemical analysis of citrus wine with treated and untreated pectic enzyme

Citrus wine	pH	Ethanol	Acidity*	Extract	Methanol	OD_{660}	Sugar**
Enzyme-treated	3.9	13.5 %	1.11 %	4.17 %	0.05 %	0.017	1.22 %
Untreated	3.8	14.3	0.96	3.12	0.04	0.052	0.55

* Total acidity is expressed as citric acid.
** Residual reducing sugar is expressed as g/100ml after fermentation.

sediment was proceeded very slowly during aging. Optical density of enzyme treated wine is lower than the untreated as shown in Table 4.the appearance of enzyme treated citrus wine was transparent after fermentation, but became to colored slowly during aging. In U. S., about 0.1% of Pectionol-O or equivalent amount of other pectice enzyme should be added for clarification of wine from orange produced in California (Amerine et al. 1972).

If a certain fruit is particularly rich in pectin, the addition of pectic enzymes to the crushed fruit may elevate the addition of pectic enzymes to the crushed fruit may elevate the methanol content since pectin esterase hydrolyzes the methoxyl groups from pectin molecules. Liberation of methanol from the hydrolysis of pectin in citrus wine was found to be very small as shown in Table 4.

During aging, citrus wine was being colored to dark brown. Two general mechanisms for browning of wine have received the most consideration: melanoidin formation by sugar-amino acid(Maillard) interaction and oxidation of phenolic compounds to brown pigments either enzymatically or nonenzymatically(Reed 1975). Several works demonstrated that increasing temperature, pH, and oxygen as well as time increased browning, while SO_2 retarded but did not prevent browning. Therefore, further studies on preventing browning of citrus wine such as treatment of a wine fining agent, inactivation of enzyme after clarification or low level of enzyme concentration, oxygen-free conditions of wine, removal of polyphenols, and preservation of wine at low temperature would be necessary.

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사 사

본 연구는 1986년도 공업진흥청 국립공업시험원의 공업기술연구생 지원금으로 이루어 졌으며, 연구에 많은 협조를 하여주신 박 인규 제주지방공업시험소장과 김 주봉 시험계장님께 감사드립니다. 또한 실험에 도움을 준 (주)현대화성 오 기만 군, 제주대학교 농화학과 김 상무군에게도 감사드린다.

摘 要

투명하고 방향성이 좋은 감귤발효주를 생산하기 위하여, 감귤중에 함유하고 있는 고분자물질을 분해할 수 있는 효소생산성 유용균주를 제주도의 토양중에서 분리 선발하였으며, 이 균주가 생산하는 효소의 생산성 및 효소의 특성과 감귤발효주 제조중에 처리하여 그의 효과를 검토하였다. 선발균주는 형태학적 제특성을 검토한 결과 *Aspergillus niger* CCM−4로 동정하였다. 밀기울 또는 감귤피를 탄소원으로한 고체배양에서 30°C, 3일간 배양하므로써 펙틴분해효소의 생산이 가능하였으며, 배지의 pH와 그외의 요인등은 효소생산에 큰 영향을 주지 않았다. 조효소액의 반응최적조건은 pH 4.0, 온도 40°C였으며, 50°C에서 1시간처리 후에도 효소의 안정성을 유지하고 있었다. 조효소를 감귤발효주 제조중에 첨가하므로써 효소처리를 하지않은 감귤주에 비하여 투명한 감귤주를 얻을 수 있었으며, 현탁물의 침전도 빨리 일어나 효소처리가 감귤주 제조에 유효함을 알 수 있었다. 감귤주의 숙성중에 일어나는 갈변현상을 방지할 수 있는 연구는 차후 검토과제로 판단된다.