Appl Microbiol Biotechnol (1991) 34:586–590
0175759891000313

Applied and Microbiology Biotechnology

Stimulation of protease production by *Aspergillus oryzae* with oils in continuous culture

Yaichi Fukushima[1], Harumichi Itoh[2], Tetsuro Fukase[2], and Hiroshi Motai[1]

[1] Research and Development Division, Kikkoman Corporation, 399 Noda, Noda City, Chiba 278, Japan
[2] Research and Development Division, Kurita Industries Ltd., Wakamiya, Morinosato, Atsugi City, Kanagawa 243-01, Japan

Received 7 August 1990/Accepted 24 September 1990

Summary. The effect of soy sauce oil and various other oils on protease production by *Aspergillus oryzae* NISL 1913 was studied in chemostat cultures (dilution rate = 0.02 h^{-1}). Soy sauce oil was consumed as a carbon source by the cells and also accelerated protease production. When soy sauce oil was used as sole carbon source, the specific protease production rate was 2.89 protease units·(mg dry weight of mycelium)$^{-1}$·h^{-1}, which was threefold higher than that with starch. The specific protease production rate with linoleic acid, oleic acid, Tween 80 and soybean oil exhibited similar values to that with soy sauce oil but the fatty acids with carbon chains shorter than six, such as caproic acid and acetic acid, did not stimulate protease production. The oils did not cause an increase in other exocellular enzymes such as α-amylase, indicating that the protease production was selectively stimulated by the oils.

Introduction

We have already succeeded in operating a chemostat culture of the salt-tolerant fungus *Aspergillus oryzae* NISL 1913 for more than 50 days without any bacterial infection in the presence of 10% NaCl (Fukushima et al. 1989). Previously, starch was used as a carbon source and soy sauce oil was used as an antifoam. In successive experiments, it was noticed that soy sauce oil was not only consumed by the cells, but also markedly promoted protease production. Since, soy sauce oil is cheaply obtained as a by-product of the soy sauce industry (Uchida and Mogi 1971), its use as a carbon source would decrease the cost of protease production.

Many workers have reported that fatty acids, oils and surfactant promoted the production of exocellular enzymes (Yamamoto et al. 1964; Reese and Maguire 1969, 1971; Tangnu et al. 1981) and some substances such as riboflavin (Kojima et al. 1972). They suggested that their effects were due to derepression, induction or stimulation of secretion. However, there have been few reports concerning the effect of oils on protease production by *Aspergillus* sp. as well as oil utilization. In this paper, we have investigated the effect of soy sauce oil, other types of oils and fatty acids on protease production by *A. oryzae* NISL 1913.

Offprint requests to: Y. Fukushima

Materials and methods

Chemostat culture conditions. Chemostat cultures of *A. oryzae* NISL 1913 were carried out as described previously (Fukushima et al. 1989) except that the broth was harvested by using a weight controller system with a balance (Mettler, Greisensee, Switzerland) and a micro-computer (Epson, Tokyo, Japan) in order to keep a constant volume in the fermentor and diminish plugging in the tube attached to the fermentor (Fig. 1). Preculture medium contained soluble starch, 3.5%; polypeptone, 2.0%; KH_2PO_4, 0.5%; $MgSO_4 \cdot 7H_2O$, 0.5%; $CaCl_2 \cdot 2H_2O$, 0.026%; $ZnCl_2$, 0.01%; yeast extract, 0.03%; NaCl, 10% in tap water. The chemostat was started after preculture for 72 h with stirring at 300 rpm with 1 vvm of air. In the chemostat culture the dissolved oxygen was maintained above 50% of air saturation with 0.02 vvm oxygen and 0.1 vvm air, and stirring at 400–550 rpm. The culture was controlled at 30° C and pH 6.5 by adding 5 N H_2SO_4 or 5 N NaOH.

When the effect of soy sauce oil concentration on protease production was studied, the first feed medium (start up) contained the same components as those of the preculture medium except that 1.30% isolated soy protein (ISP) was added as a nitrogen source, and 1.25% soluble starch and 0.40% soy sauce oil were included. After the protease activity reached a steady state (after 10 days), feed medium, containing the same components as preculture medium except that 2.60% soybean flour as the nitrogen source, and 1.0% soluble starch and various concentrations of soy sauce oil as carbon sources were added, was supplied continuously. The dilution rate (D) was fixed at D = 0.02 h^{-1}, the optimum found in previous work (Fukushima et al. 1989). The effect of NH_4^+ on protease production was studied as follows: after protease production reached a steady state, NH_4Cl was transiently added to the broth in the fermentor to a concentration of 800 ppm $NH_4^+ - N$, then the protease activity and residual $NH_4^+ - N$ were determined. The feed medium, containing the same components as preculture medium except that 1% soybean flour as the nitrogen source and 1% soy sauce oil as the carbon source were included, was supplied at D = 0.03 h^{-1}.

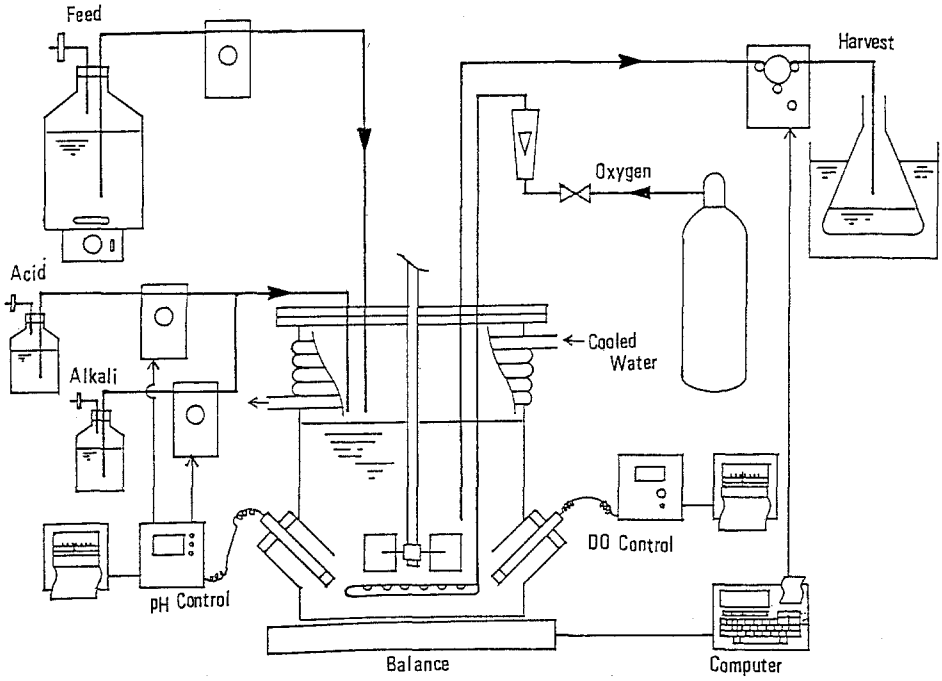

Fig. 1. Scheme of chemostat culture: Do, dissolved oxygen

Batch culture conditions. Spores of *A. oryzae* (NISL 1913) were inoculated into a 500-ml erlenmeyer flask containing 100 ml medium comprising the same components as the preculture medium except that 1% soybean fluor as a nitrogen source and 1% oil or 2% sugar as carbon sources were included. The spores were cultured on a rotary shaker at 30° C for 72 h.

Analytical methods. Protease activity at pH 7.0 was measured according to Fukushima et al. (1989). One unit of the protease activity (PU) was defined as the amount that catalysed the release of 1 μg tyrosine per minute. α-Amylase activity was measured by the method reported by Ushijima et al. (1990). One unit (U) of α-amylase was defined as the amount that hydrolysed 1 mg starch per 30 min. Total organic carbon (TOC) was determined with a TOC analyser (Shimadzu, Kyoto, Japan). Cell yield [cell dry weight (DW)/TOC] was calculated by dividing the DW (mg/ml) by the consumed TOC (mg/ml), measured by subtracting the residual TOC in the broth from TOC in the feed medium. The NH_4^+ concentration was measured by the method reported by Tobor (1970).

Materials. Silicon oil (KM-72F) was purchased from Shin-etsu Kagaku (Tokyo, Japan). The ISP (Purina 630J) was from Fuji Purina, Tokyo, Japan. The defatted soybean flour (Puffmin SM) and soy sauce oil were prepared by Kikkoman, Noda, Japan.

Results and discussion

Effect of soy sauce oil on protease production in chemostat culture

Figure 2 shows the time course of protease production in a carbon-limited chemostat culture with soy sauce oil as the carbon source. With increasing soy sauce oil concentration (0.65–2.0%), protease production increased and reached 2500 PU/ml. The averages of the various parameters in the steady state at various concentrations of soy sauce oil in the chemostat culture are shown in Fig. 3. With increasing soy sauce oil concentration up

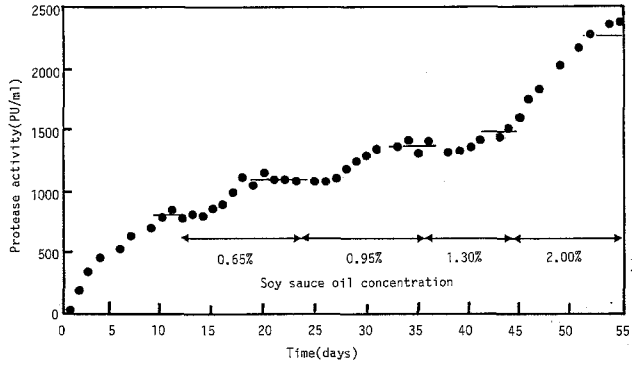

Fig. 2. Effect of soy sauce oil on protease production in a chemostat culture at dilution rate (D) = 0.02 h^{-1}. PU, protease units

to 2.0%, the mycelium DW increased from 14 mg/ml to 22 mg/ml and the specific protease production rate also increased from 1.50 to 2.20 PU·(mg DW)$^{-1}$·h^{-1}. In contrast, the residual ammonia decreased.

The increase in the specific protease production rate might have been due to a decrease in nitrogen catabolite repression by decreasing NH_4^+. In order to investigate the effect of NH_4^+ on protease production, NH_4Cl was transiently added to the chemostat culture at a steady state of carbon limitation (Fig. 4). However, the level of protease activity was not varied by changing the NH_4^+ concentration, indicating that the protease production was not repressed by excess ammonia in a carbon-limited chemostat.

Comparison of soy sauce oil and sugar as carbon sources for protease production in chemostat cultures

In order to investigate the effect of soy sauce oil on protease production, we tested various carbon sources including carbohydrate as limiting nutrients in carbon-

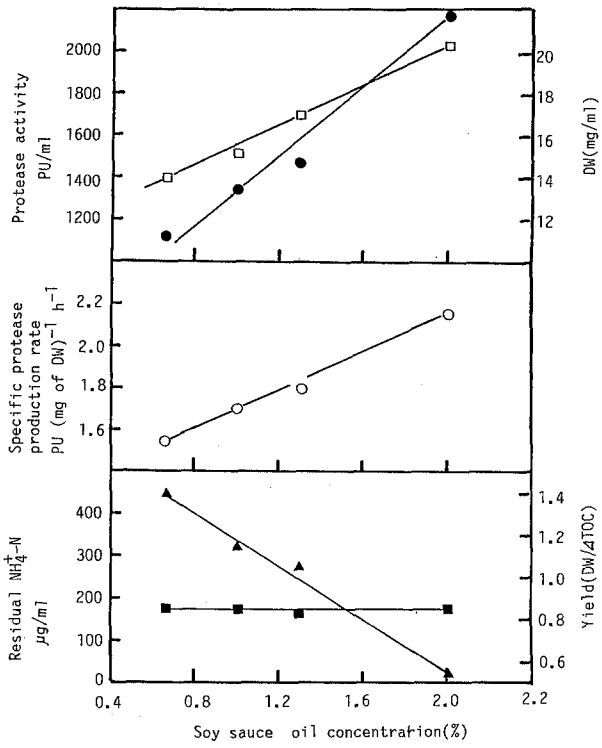

Fig. 3. Effect of soy sauce oil concentration on protease production and cell growth, measured as cell dry weight (DW) in chemostat culture. The DW, protease activity, residual ammonium and residual total organic carbon (TOC) were measured (see text) at the steady states of various concentrations of soy sauce oil in chemostat culture (see Fig. 2): □, DW; ●, protease activity; ○, specific protease production rate; ▲, residual ammonia; ■, cell yield against consumed TOC

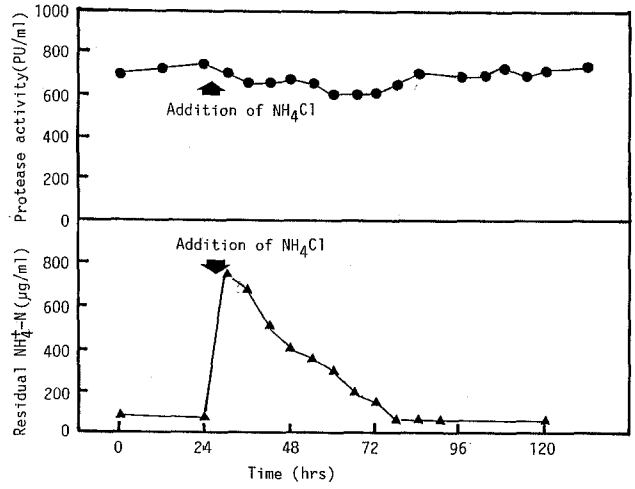

Fig. 4. Effect of excess ammonium addition on protease production at steady state in carbon-limited chemostat culture at $D = 0.03\ h^{-1}$

limited chemostat cultures (Table 1). When starch was used as the sole carbon source, silicon oil was used as an antifoam. The specific protease production rate with soy sauce oil as the sole carbon source was 2.89 $PU \cdot (mg\ DW)^{-1} \cdot h^{-1}$, which was threefold higher than with starch as the sole carbon source. The specific α-amylase production rate with starch as the sole carbon source was 38.0 $U \cdot (mg\ DW)^{-1} \cdot h^{-1}$, threefold higher than when soy sauce oil was the sole carbon source. This indicates that starch was an inducer of α-amylase production, in agreement with published data (Erratt et al. 1984). When starch was used in the presence of 0.65% soy sauce oil, the specific α-amylase production rate was almost the same as that with starch in the absence of soy sauce oil. Consequently, it was concluded that soy sauce oil did not accelerate the secretion of α-amylase and caused selective acceleration of protease production.

Kojima et al. (1972) reported that fatty acids accelerated riboflavin production because of a derepression effect, and Yamamoto et al. (1964) reported that the saturated fatty acids accelerated amylolytic enzyme production by *Endomyces* sp. because of the same effect. It is probable that the effect of soy sauce oil on protease production was due to derepression because the specific protease production rate with soy sauce oil used as the sole carbon source was higher than that with both soy sauce oil and starch (Table 1).

Comparative effect of various fatty acids on protease production in batch cultures

Uchida and Mogi (1971) reported that soy sauce oil was derived from soybean oil (triglycerides) hydrolysed to fatty acids in a brewing process by lipase produced by the *koji* mold. Soy sauce oil contains 83–90% fatty acids, composed of linoleic acid (55–60%), oleic acid (15–20%), palmitic acid (11–17%), linolenic acid (6–9%)

Table 1. Comparative effect of carbon sources on protease production in C-limited chemostat cultures

Carbon source	Protease activity (PU/ml)	Dry weight (DW) (mg/ml)	Specific protease production rate [$PU \cdot (mg\ DW)^{-1} \cdot h^{-1}$]	Specific α-amylase production rate [$U \cdot (mg\ DW)^{-1} \cdot h^{-1}$]
Soy sauce oil (1.8%)	1460	10.1	2.85	11.3
Starch (1.3%) + oil (0.65%)	1000	10.0	2.00	38.0
Starch (3.0%)[a]	620	13.5	0.92	36.0

The components of the feed medium were the same as those of preculture medium except for adding 1.30% isolated soy protein (ISP) as a nitrogen source and soy sauce oil and/or starch as the carbon source: PU, protease units; U, α-amylase units

[a] Silicon oil was used as an antifoam instead of soy sauce oil

and stearic acid (2–3%). In order to investigate which component is effective in protease production, various fatty acids were compared in batch cultures.

As shown in Table 2, the protease activity (PU/ml) with linoleic acid, oleic acid, myristic acid and soy sauce oil were higher than that with starch. Therefore the effect of soy sauce oil could be due to the fatty acids it contains, those with carbon chains longer than 14 being effective for protease production. Growth was entirely inhibited by capric acid, caproic acid, propionic acid and acetic acid, all with carbon chain lengths shorter than ten. These data are consistent with the results of Sheu and Freese (1972). Therefore, it was not good to investigate the effect of the carbon chain length, to which growth inhibition was related, on protease production by batch culture.

Comparative effect of various oils on protease production in chemostat cultures

As shown in Table 3, the protease activity (PU/ml) and the specific protease production rate with soy sauce oil, soybean oil, linoleic acid and oleic acid reached almost the same level, 1270–1460 PU/ml and 2.49–2.89 PU·(mg DW)$^{-1}$·h^{-1}, respectively, in chemostat culture. Fatty acids with carbon chains shorter than six did not stimulate protease production more than starch. However the growth yield was independent of the carbon chain length of the fatty acids in the carbon-limited chemostat culture. These data differed from the results obtained in batch cultures (Table 2), indicating that cytotoxicity was neutralized in the chemostat culture because the residual fatty acid was kept at low level and the feed medium was supplied at a slow rate. If the protease was efficiently produced through derepression when fatty acids were used, intermediates in the β-oxidation pathway, such as caproic acid and acetic acid, might stimulate protease production at similar levels to those with oleic acid and linoleic acid. However, the specific protease production rate with caproic acid and acetic acid was about one quarter of that obtained with oleic acid and linoleic acid. Therefore, it was concluded that the stimulation of protease was not caused by derepression and that fatty acid chain length was important in protease production.

Table 2. Comparative effect of fatty acids and sugar on protease production in batch cultures

Carbon source	Protease (PU/ml)
Soy sauce oil	230
Linoleic acid (C_{18})	270
Oleic acid (C_{18})	270
Stearic acid (C_{18})	250
Myristic acid (C_{14})	200
Capric acid (C_{10})	0
Caproic acid (C_6)	0
Propionic acid (C_3)	0
Acetic acid (C_2)	0
Starch	110

Culture conditions: See text

Many workers have reported that the production of various enzymes is stimulated by the addition of surfactants such as Tween 80 (Reese and Marguire 1969, 1971; Tangnu 1981; Stuzenberger 1987), which is an ester of monooleic acid and polyoxyethylene sorbitan. When Tween 80 was used as the sole carbon source, both protease activity (PU/ml) and DW were lower than those with oleic acid. However, the cell yield against the consumed TOC and the specific protease production rate were almost the same as those with oleic acid. The residual TOC using Tween 80 was about 8.3 mg·ml^{-1}, which is higher than that using other carbon sources (2.0–2.5 mg·ml^{-1}), indicating that the polyoxyethylene sorbitan of Tween 80 might not be consumed and might remain in the broth. Thus, the effect of oleic acid and Tween 80 on protease production were almost the same; stimulation of protease production was not related to a surfactant such as a fatty acid sugar ester but to a fatty acid of long carbon chain length.

Effect of Tween 80 on protease and α-amylase production by washed mycelium

To investigate the effect of oil in batch culture, Tween 80 was used because preliminary experiments had indi-

Table 3. Effect of oils on protease production in C-limited chemostat cultures

Carbon sources	Protease activity (PU/ml)	DW (mg/ml)	Specific protease production rate [PU·(mg of DW)$^{-1}$·h^{-1}]	Yield [mg DW/mg ΔTOC]
Soy sauce oil (1.8%)	1460	10.1	2.89	0.65
Soybean oil (1.5%)	1390	11.0	2.53	0.72
Linoleic acid (1.5%)	1390	10.0	2.62	0.65
Oleic acid (1.5%)	1270	10.5	2.49	0.68
Caproic acid (2.0%)	350	10.4	0.67	0.68
Acetic acid (3.0%)	380	10.4	0.73	0.68
Tween 80 (1.8%)	664	5.5	2.41	0.68
Starch (3.0%)	620	13.5	0.92	0.81

The feed medium contained the same components as those in preculture medium except that various oils were used as carbon sources such that the total organic carbon concentration of the oils or starch in the feed medium were about 1.2% and 1.3% ISP was used as a nitrogen source

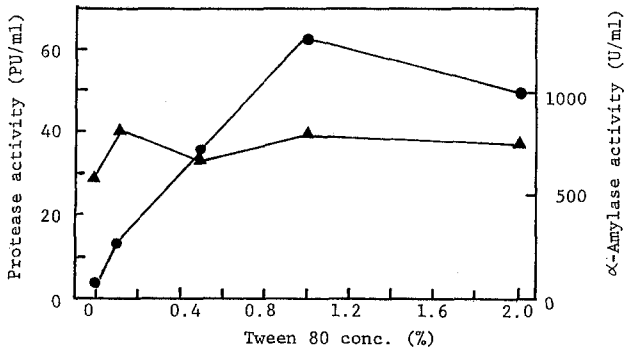

Fig. 5. Effect of the Tween 80 concentration on protease and α-amylase production by washed cells. After 22 h cultivation in preculture medium lacking NaCl, cells were washed with 0.05 M phosphate buffer containing 0.001% $CaCl_2 \cdot 2H_2O$ and 0.003% $MgSO_4 \cdot 7H_2O$, transferred to a medium containing the indicated concentration of Tween 80 in the same buffer as the washing buffer and incubated for 7 h. The initial DW was 5.1 mg/ml: ●, protease activity; ▲, α-amylase activity

cated that the cytotoxicity of Tween 80 might be lower than oleic acid (data not shown). Figure 5 shows the effect of Tween 80 on protease and α-amylase production by washed mycelium. Although the protease was poorly produced in the absence of Tween 80, it increased with increasing Tween 80 concentration up to 1.0%. In contrast, α-amylase activity was independent of the Tween 80 concentration. This supports the results described in Table 1 showing that oil selectively stimulated protease production. When cycloheximide was added to the medium in the presence of Tween 80, the protease was not secreted into the medium, indicating that the protease, which was synthesized de novo, was secreted only in the presence of Tween 80 (Drucker 1972).

From these results, it is very advantageous to use soy sauce oil as a carbon source and antifoam for protease production. We should investigate further the regulation of synthesis of the protease and its secretion.

Acknowledgements. We thank Prof. R. Matsuno of Kyoto University and Prof. K. Toda of Tokyo University for their encouragement and Dr. M. Kamekura and Dr. M. Suzuki of the Kikkoman Corporation for their kind advice. Thanks are also due to Mr. K. Okada for technical assistance.

References

Drucker H (1972) Regulation of exocellular protease in *Neurospora crassa*. Induction and repression of enzyme synthesis. J Bacteriol 110:1041–1049

Erratt JA, Douglas PE, Moranelli F, Seligy VL (1984) The induction of α-amylase by starch in *Aspergillus oryzae*: evidence for controlled mRNA expression. Can J Biochem Cell Biol 62:678–689

Fukushima Y, Itho H, Fukase T, Motai H (1989) Continuous protease production in a carbon-limited chemostat culture by salt tolerant *Aspergillus oryzae*. Appl Microbiol Biotechnol 30:604–608

Kojima I, Yoshikawa H, Okazaki M, Terui Z (1972) Studies on riboflavin production by *Eremothecium ashbyii*. J Ferment Technol 50:716–723

Reese ET, Maguire A (1969) Surfactants as stimulants of enzyme production by microorganisms. Appl Microbiol 17:242–245

Reese ET, Maguire A (1971) *Aureobasidium pullulans* as a source of sucrase. Can J Microbiol 17:329–332

Sheu GW, Freese E (1972) Effects of fatty acids on growth and envelope proteins of *Bacillus subtilis*. J Bacteriol 111:516–524

Stutzenberger FJ (1987) Component-specific stimulation of cellulase secretion in *Thermomonospora cuvata* by the surfactant Tween 80. J Appl Bacteriol 63:239–244

Tangnu SK, Blanch HW, Wilke CR (1981) Enhanced production of cellulase, hemicellulase and β-glucosidase by *Trichoderma reesei* (Rut C-30). Biotechnol Bioeng 23:1837–1849

Tobor CW (1970) The determination of the NH_4^+ with the use of glutamate dehydrogenase. Methods Enzymol 17:955

Uchida K, Mogi K (1971) Fatty acid composition of *shoyu* (soy sauce) oils and their relation to those of the raw materials. Choumi Kagaku (Jpn) 18:466–471

Ushijima S, Nakadai T, Uchida K (1990) Further evidence in the interspecific protoplast fusion between *Aspergillus oryzae* and *Aspergillus sojae* and subsequent haploidization, with special reference to their productivity of some hydrolyzing enzymes. Agric Biol Chem 54:2393–2399

Yamamoto T, Hattori F, Takatsu H (1964) The effect of lipids on amylolytic enzyme production by *Endomyces spec.* Bull InstXX