The Effect of Lipids on Amylolytic Enzyme Production by *Endomyces* sp.

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During the course of an investigation of amylolytic enzyme production using *Endomyces* sp. it appeared desirable to use media containing rice-bran. In an effort to determine the effect of rice-bran, several components of rice-bran were added to the media. It might be concluded from the results of these investigations that rice oil was effective for the production of amylolytic enzyme. The other vegetable and animal oils were also effective for production of the enzyme. Moreover, it has been found that rice oil in the medium was assimilated. Lipase was produced a little in the medium by the microorganism. The addition of mixed fatty acids which were obtained from rice oil by saponification was effective for the amylolytic enzyme poroduction and additional fatty acids were utilized.

INTRODUCTION

In 1960, Y. Hattori[1)2)3)] found that a sole strain, *Endomyces* sp. IFO 0111, produced an extracellular amylolytic enzyme which was proved to be useful for the production of glucose from starch. Since, amylolytic enzyme production using *Endomyces* sp. has been developed in industry by the application of submerged culture,and then, in our country, it used hydrolysis of starch as well as *Rhizopus* one.

The authors have tried to increase the yield of the enzyme by the improvement of medium and strain. As the results of the experiments on medium, the interesting facts were found that the medium containing lipids gave good yield of the enzyme, and that the lipids were almost assimilated by the microorganism.

In the fermentation industry, animal and vegetable oils from various sources have been used generally as antifoam agents. Only a few of fermentations use fats to increase the yield, for example the patent in which addition of fat was effective for production of erythromycin[4)], and a considerable number of papers and patents have been presented on the role of oils in penicillin production. Stefaniak, Gailey, Jarvis and Johnson[5)] suggested that oils acted primarily as foam-breaking substances, thereby enhancing the aeration and penicillin production. Goldschmidt and Koffler[6)] reported that small amounts of unsaturated oils greatly increased the penicillin yield in shaking flasks inoculated with spores. Colingsworth[7)] claimed in a patent that an increase in penicillin yield of more than 50 per cent could be obtained if 0.1 per cent of suitable oil was added to the fermentation medium shortly after inoculation. Lard oil and corn oil were claimed to be most suitable oils for the stimulation of penicillin production. S. Yasuda[8)] reported that soybean oil was very effective for the penicillin production and

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that the oleic acid itself also increased the yield of penicillin. Recently it has been reported that natural oils with a high content of unsaturated fatty acid and oleic acid itself, when added 2 per cent (v/v) to suitable fermentation media, increase the yield of citric acid by about 20 per cent, and that the lipids which improve the yield of citric acid have no effect on the dry weight of mycelium[9]. It has been found that the oleic acid as well as biotin stimulates the growth[10] of glutamic acid producing bacteria and increases the yield of glutamic acid[11].

But there is no report that the microorganism shows favorable growth in medium containing 10 per cent of fat, and especially that the assimilation of fat has some relation to enzyme synthesis. It seemed reasonable to assume that the fats have been used scarcely as fermentation medium because it was insoluble in water and it was more expensive than other carbon sources. It may be considered natural that the utilization of carbohydrate will be studied in future fermentation industry. In order to make it clear, it is necessary to study the physiological and physical effects on microorganism by the additional fats. The authors intend to explain the relation of amylolytic enzyme synthesis, and assimilation of fats and fatty acids, and intend to find the new facts of this fermentation. This report describes the several results obtained in the study of the amylolytic enzyme production by *Endomyces* sp.

EXPERIMENTAL

1. Cultural Conditions

Hundred milliliters of the medium was poured in 500ml. shaking flask. These were sterilized with steam for thirty minutes at 120 °C. After cooling, they were inoculated with 1 ml. of seed which was cultured in wort medium for forty eight hours at 30°C, and incubated on reciprocal shaker of 150 strokes per minute, 7.5 cm. amplitude. Seventeen liter portions of the medium were poured in 25 L. fermentor. They were sterilized with steam in the same condition as the shaking flask. The cultural conditions in fermentor were as follows: temperature at 30°C, aeration 17 L./min., agitation speed 400 r.p.m., and cultural period sixty hours. The medium composition was as follws: bouillon 2%, strach 1%, potassium biphosphate 0.1% and rice oil 3%.

2. Assay Procedures for the Amylolytic Enzyme Activity

Procedures which had been employed by Fukumoto[12] were used with some modifications. The saccharifing activity was measured by the increase in reducing power of the reaction mixture, composed of 10 ml. of 2 % of soluble starch, 5 ml. of 0.2 *M.* acetate buffer solution (pH 4.8), 4 ml. of distilled water, and 1 ml. of the enzyme solution. After incubation for thirty minutes at 40°C, 2 ml. of reaction mixture were taken, and the reducing power was determined by means of the modified Somogy's method. When the reducing power in 2 ml. of the reaction mixture was equivalent to 1 mg. of glucose, the saccharifing activity of the enzyme solution was defined as 1 unit. Cultural broth was separated into cells and other dregs by centrifuge. Supernatant solution was used as the enzyme solution.

3. Extraction of Effective Components of Rice-bran

The rice-bran (100 g.) was extracted with ethanol at 80°C for eight hours under reflux. After extraction the residue was filtered and dried by spreading. The oily brown substance was obtained from ethanol extraction after evaporating ethanol under reduced pressure. This fraction was named F. 1 and it was diluted up to 100 ml. with distilled water. These treatments are shown in Fig. 1.

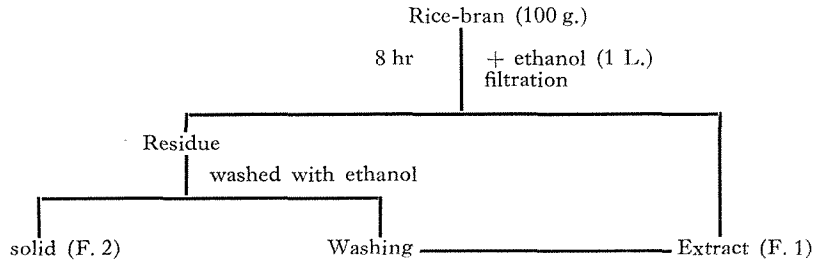

Fig. 1 Extraction of effective component from rice-bran

The oily brown substance which was extracted with ethanol from rice-bran included the solid (F. 3), the composition of which was mainly phospholipid. The F. 4 was separated from F. 1 by centrifuge and was kept at 0°C for one week. Then, F. 4 again produced a precipitate which was waxen substance. After the waxen substance was removed by centrifuge, 10 % sodium hydroxide solution was added to the supernatant F. 5 and was kept at 60°C for one hour. By this treatment, the fatty acid in F. 5 was solidified. Two per cent clay activate was added into the liquid part F. 8 after the fatty acid sodium salt F. 7 was removed by filtration. After it was kept at 80°C for one hour, it lost color. The volatile substance F. 11 was removed by steam distillation from F. 9 which was obtained by the filtation with clay activate. The F. 10 was colorless purified rice oil. These treatments are given in Fig. 2.

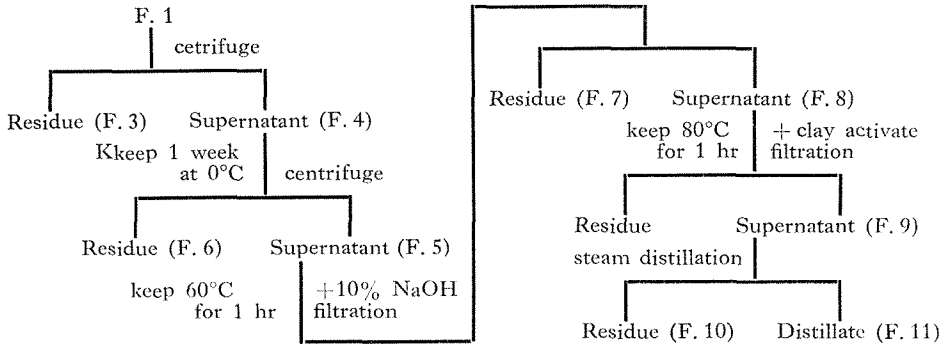

Fig. 2 Extraction of effective component of F. 1

3. Determination of Residual Oil

The residual oil in filtered broth and oil in the microorganism were extracted

by the Soxhlet extracter with ether for twenty hours, and they were weighed after removal of ether. Residual oil was determined as ether soluble substance.

4. Measurement of Respiration

The composition of materials in vessels are given in Table 1. The preparation of cells suspension was as follows: *Endomyces* sp. was cultured in wort medium for forty hours, and separated by centrifuge. The cells were suspended in 0.9 % sodium hydroxide solution. The cells concentration was 1.1 mg./ml. Measurements were made at 30°C.

Table 1. Composition of material on vessels.

Vessel	Rice oil (ml)	Acetate buffer (ml)	Cells suspension (ml)	H_2O (ml)	KOH (ml)
A	0.09	0.50	0.50	1.91	0
B	0.09	0.50	0.50	1.71	0.20

5. Assay Procedures for the Lipase Activity

The determination of lipase was made by the Nord method[13)14)], as modified by Yamada[15)]. One milliliter of enzyme solution was added into 50 ml. concical flask containing the mixture of 5 ml. of olive oil emulsion and 3 ml. of phosphate buffer (pH 4.5 - 9.0) was ready warmed at 37°C. The mixture was shaken at 37°C for four hours. After digestion, to the reaction mixture was added 20 ml. of ethanol-aceton (1 : 1) to stop the reaction and to break the emulsion. Free fatty acids in the reaction mixture were titrated with 0.1 *N* potassium hydroxide after the addition of phenolphthalein. Lipase activity was defined as the number of milliliter of 0.1 *N* potassium hydroxide solution.

The preparation of emulsion was as follows. Twenty grams of polyvinylalcohol (polymerization degree 1700) was dissolved in 1 l. of water with stirring and 5 ml. of 0.1 *N* hydrochloric acid was added. The polyvinylalcohol solution was heated at 75 - 85°C and was filtered. After cooling, 0.1 *M* of olive oil supposed to be triolieate was added and the mixture was homogenized and emulsified.

6. Preparation of Intercellular Enzyme Sampls

The cells in broth were separated by centrifuge and washed with 0.9 % of sodium chloride solution several times. The cells were ground with glass powder in a mortar after washing and were treated by two processes of freezing and melting over and again. Phosphate buffer solution (pH 4.8) was added to contain 2 % of ground cells and make it cool for 24 hours in a ice box to extract the enzyme. The mixture was separated to cells and glasspowder, and supernatant by centrifuge. This supernatant was used as intercellular enzyme.

RESULTS

1. Effect of Nitrogen Source on Amylolytic Enzyme Production

Y. Hattori found that yeast extract, poly pepton, and wheat-bran were good nitrogen

sources for the enzyme production. The present investigation was undertaken to increase yields of the enzyme by improvements of medium. Using defatted soybean, the resolvent of defatted-soybean, wheat-bran, and rice-bran for nitrogen source, the relation between their content and yield of the enzyme was examined.

Time course of the enzyme production used defatted-soybean and rice-bran are given in Table 2.

Table 2. Time course of the enzyme production

Media No.	Rice-bran (%)	Time (hrs)		
		48	72	96
1	0	3.7	4.9	5.6
2	0.5	4.3	7.0	8.2
3	1.0	4.5	8.3	9.0

Each flask contained: Deffated-soybean 3 %, soluble starch 1 %, and KH_2PO_4 0.1 %.

The enzyme activity in cultural broth of medium 1 arrived at nearly maximu value in a shorter period compared with medium 2 and medium 3. The amximum activity of the enzyme in medium 3 compared with medium 1 increased about one and half times in ninety-six hours. Relations between variation of rice-bran, defatted-soybean and enzyme production are given in Table 3.

Table 3. Relation between rice-bran, defatted-soybean and the enzyme production.

Defatted-soybean (%)	1.0	3.0	5.0	1.0	3.0	5.0	1.0	3.0	5.0
Rice-bran (%)	0	0	0	0.5	0.5	0.5	1.0	1.0	1.0
Activity (unit/ml)	5.6	5.6	5.9	8.4	8.2	7.0	9.1	9.0	8.1

Each medium contained: soluble starch 1.0 %, KH_2PO_4 0.1 %,
The enzyme activity of the medium was determined at 96 hours.

The experiment described below leaves no doubt that defatted-soybean was non-effective for the production. From a consideration of increasing of viscosity on account of overgrowth of the microorganism, it seemed more reasonable to conclude that the decrease of the enzyme activity in defatted-soybean rich media was due to a reduced agitation effect.

The next investigation was undertaken to confirm the effect of rice-bran on the amylase production using resolvent of defatted-soybean instead of defatted-soybean. The preparation of resolvent of defatted-soybean is as follows. Defatted-soybean, 150 g., was added to 500ml. of distilled water and the mixture was heated at 120°C with steam in a autoclave. The mixture of 40 g. of Bioplase (protease) and 200 ml. of distilled water was filtered. The two solutions were diluted up to 1. with distilled water, allowed to settle at 40 - 45°C for three days, and finally, boiled at 100°C.

The time course of the amylase production used resolvent of defatted-soybean and rice-bran is shown in Table 4.

It has been found from these results that the amylase activity was increased

Table 4. Time course of the enzyme production used resolvent of defatted-soybean.

Rice-bran (%)	Time (hr)	Resolvent of defatted-soybean (%) 0.5% 48	72	96	1.0% 48	72	96
0		2.7	3.2	3.5	2.6	3.5	4.6
0.5		4.4	5.6	6.2	3.4	4.6	5.3
1.0		5.2	7.1	8.2	3.7	5.5	5.9

Each medium contained: Soluble starch 1.0 %, KH_2PO_4 0.1 %

Table 5. Relation between resolvent of defatted-soybean and rice-bran, and the enzyme production.

Resolvent of defatted-soybean (%)	0.5	1.0	3.0	0.5	1.0	3.0	0.5	1.0	3.0
Rice-bran (%)	0	0	0	0.5	0.5	0.5	1.0	1.0	1.0
Activity (unit/ml.)	3.5	4.6	4.9	6.2	5.3	4.4	8.2	5.9	3.6

Each medium contained as follows: Soluble starch 1 %, KH_2PO_4 0.1 %
The enzyme activity of broth was determined at 96 hours.

according to increase of the amount of rice-bran and that the amylase production was stimulated by addition of rice-bran.

Relation between variation of rice-bran, resolvent of defatted-soybean and amylase production is given in Table 5.

The results of the studies in using resolvent of defatted-soybean support the hypothesis that the rice-bran was effective for the enzyme production, but in the view point of the improvement of media, the resolvent of defatted-soybean has not always been proved successful. The resolvent of deaftted-soybean was somewhat effective in the rice-bran deficient media, but it inhibited the production of enzyme in the media containing rice-bran.

In the similar manner, wheat-bran was used for nitrogen sourse.

Table 6 gives time course of the enzyme production used wheat-bran and rice-bran.

When the proportion of the rice-bran increased, the enzyme production started later than in medium 1, but maximum value of enzyme activity increased evidently. For example, maximum value of the enzyme activity in medium 4 was three times greater than in medium 1. In Table 7 are shown the relations between variation of

Table 6. Time course of the enzyme production used wheat-bran and rice-bran.

Medium no.	Rice-bran	Time (days) 2	4	6	8	11	15
1	0.5 %	3.2	9.9	11.6	12.1	12.9	
2	1.0 %	1.2	10.1	12.5	13.0	13.5	
3	3.0 %	0.4	5.6	13.6	15.3	18.3	18.4
4	5.0 %	0	2.8	18.3	22.9	28.3	28.9

Each medium contained as follows: wheat-bran 3 %, soluble starch 1 % KH_2PO_4 0.1 %.

Table 7. Relation between variation of rice-bran and wheat-bran concentration, and the enzyme production.

Whea-tbran (%)	Rice-bran (%)								
	0	1.0	3.0	5.0	7.5	10.0	12.5	15.0	20.0
0			13.4	22.4	28.7	32.2	37.5	30.7	6.4
1.0		9.0	15.7	23.5	30.7	38.5	43.4	28.0	0
3.0	11.5	13.4	18.6	28.4	33.6	31.3	24.5	22.5	0
5.0	12.5	17.4	22.8	24.0	30.5	20.2	11.0	3.8	
7.5	13.2	20.0	14.8	11.3	10.5	7.4	0	0	
10.0	14.0	11.3	7.0	5.2	0	0	0	0	
12.5	9.2	6.2	0	0	0	0			
15.0	4.5	0	0	0					
17.5	0	0							

Each flask contained : soluble starch 1 %, KH_2PO_4 0.1 %
The cross-reference of a each additional value of wheat-bran and rice-bran showed the enzyme activity (unit/ml.) in this proportional medium. The enzyme activities were determined on the seventh, ninth, eleventh, and forurteenth day after inoculation and their maximum value was described.

rice-bran, wheat-bran concentration and the enzyme production. The data on the enzyme production reported herein indicate that the medium containing rice-bran 12.5 % and wheat-bran 1.0 % gave the highest activity, and that rice-bran was more effective than wheat-bran for the enzyme production.

The effect of defatted-soybean, resolvent of it, wheat-bran, and rice-bran as the nitrogen source on the amylase production were examined in the above experiments. The effects of corn-meal, corn-steep-liquor, and malt were examined in a similar manner, but the detailed data were omitted in this paper. The results of these investigation indicated that rice-bran was suitable nitrogen source on the enzyme production, too. It may be concluded from the results of these studies described above that rice-bran was the most excellent nitrogen source on the enzyme production.

2. Investigation of Effective Component of Rice-bran

In view of the above facts, it has been found that rice-bran was very effective for the enzyme production. The present experiments were initiated in order to investigate the effective components in rice-bran. At first two fractions were prepared. The data on two components reported herein evidently indicate that an effective component in rice-bran existed in the fraction extracted with ethanol. The results of this experiment are shown in Table 8.

The enzyme activity of medium 1 was given 11.5 units/ml., but the activity of medium 6 showed 22.3 units/ml., namely, the yield of the enzyme increased by a factor of two. The enzyme was not produced in medium 3. It seemed reasonable to assume that the microorganism did not grow because of the lack of nitrogen source. The enzyme activity of medium 9 reached 19.6 units/ml. which was lower than 28.7 units/ml. of medium 2. In this case, it seemed reasonable to consider that the effective substance for the enzyme production decomposed during hot extraction.

Table 8. Effect of F. 1 and F. 2 on the enzyme production.

Medium NO.	Wheat-bran (g)	Rice-bran (g)	F. 1 (ml)	F. 2 (g)	Activity (u/ml)
1	3.0				11.5
2		7.5			28.7
3			7.5		0
4				5.4	4.3
5	3.0	7.5			27.3
6	3.0		7.5		22.3
7	3.0			5.4	9.7
8	3.0		7.5	5.4	22.4
9			7.5	5.4	19.6

Each flask contained : soluble starch 1.0 %, KH_2PO_4 0.1 %.
Seven and half milliliter of F. 1 and 5.4 g. of F. 2 were extracted from 7.5 g. of rice-bran. The enzyme activities were determined on the seventh, ninth, eleventh, and fourteenth day after incoculation and their maximum value was described.

Table 9. Effect of each fraction on the enzyme production.

F. No.	Activity (u/ml)	F. No.	Activity (u/ml)
1	18.9	8	18.0
3	8.9	9	18.0
4	18.7	10	12.0
5	18.8	11	16.5
6	15.0		
7	11.6	Control	11.1

Basal media contained : Wheat-bran 3 %, soluble starch 1 % and KH_2PO_4 0.1 %.
Each fraction was added 3 % in basal media. Cultural period was ten days.

In view of the above facts, the reasonable conclusion to be drawn from the available data is that the effective components may be extracted from rice-bran with ethanol; so F. 1 was fractionated over again by the method described above in order to investigated the effective substance in F. 1. *Endomyces* sp. was cultured in medium to which one of the fractions was added in the basal medium. The results of this experiment are given in Table 9.

The data on the effective component described above indicated that the effective substance of F. 1 remained in the liquid part; from F. 1 to F.4, F.5, F.8, F.9, and F. 10, only the precipitate F. 6 gave a little effect. The soap (F. 7),the phospholipid (F. 3) and the volatile substance (F. 11) were not effective.

3. Effect of Oils on the Enzyme Production

On the basis of these data, it seemed reasonable to assume that the effective component of rice-bran was rice oil. So this investigation was undertaken to examine the effect of rice oil on the enzyme production, and to study the effect of the other several oils. At first, the relation between rice oil and the enzyme production was studied using media in which various amounts of rice oil were added to defatted-soybean and

wheat-bran. Time course of the enzyme production which defatted-soybean was used as nitrogen source are given in Fig. 3.

The amylolytic enzyme activity of rice oil deficient medium was 5.8 units/ml., but the activity of medium containing rice oil 1 % showed 21 units/ml. Moreover, the enzyme activity of medium containing 3 or 5 % of rice oil increased to 50 units/ml., *i.e.* about ten times increase compared with rice oil deficient medium.

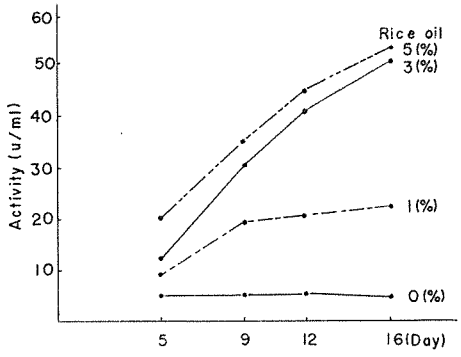

Fig. 3. Time course of the enzyme production.
Each flask contained defatted-soybean 3.0%, soluble starch 1.0% and KH_2PO_4 0.1%.

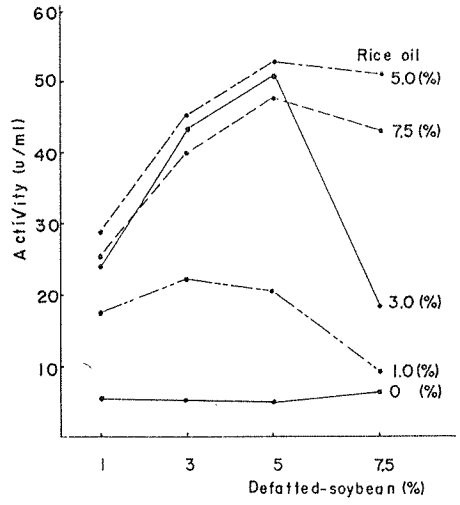

Fig. 4. Effect of rice oil on the enzyme production.
Each medium contained soluble starch 1.0%, KH_2PO_4 0.1%. Cultural period was twelve or sixteen days.

The next investigation was undertaken to study the relation between variation of rice oil and defatted-soybean concentration. These results are given in Fig. 4.

When the concentration of defatted-soybean was 5 %, the enzyme activity showed the highest value in every concentration of rice oil, and increased according to the increase of rice oil up to 5%. But when the rice oil went over 5%, the enzyme production decreased. This phenomenon was observed in 1, 3, and 7.5 % defatted-soybean. In the case of using defatted-soybean for nitrogen source, the most favorable composition of medium was as follows: rice oil 5 %, defatted-soybean 5 %, soluble starch 1 %, and potassium biphosphate 0.1 %. In the experiments described below, wheat-bran was used for nitrogen source in place of defatted-soybean in the above experiments. Time course of the enzyme production in which wheat-bran was used is given in Fig. 5.

Rice-oil deficient medium for control, the enzyme activity showed 11.0 units/ml. after eight or twelve days. The activity of medium containing rice oil showed a difference by the fourth day, but the effect of rice oil appeared on the eighth and twelfth day. In the case of rice oil concertration 3 %, the enzyme activity showed three times compared with rice oil deficient medium. When the medium containing rice oil 5 % was used, the viscosity of the broth increased by the overgrowth of the

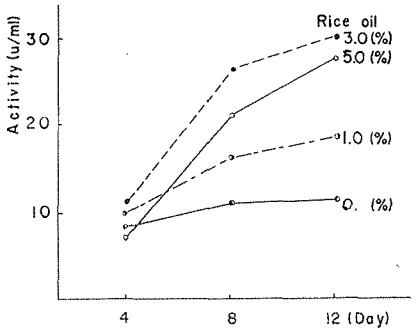

Fig. 5. Time course of the enzyme production.
Each flask contained wheat-bran 3.0%, soluble starch 1.0% and KH_2PO_4 0.1%.

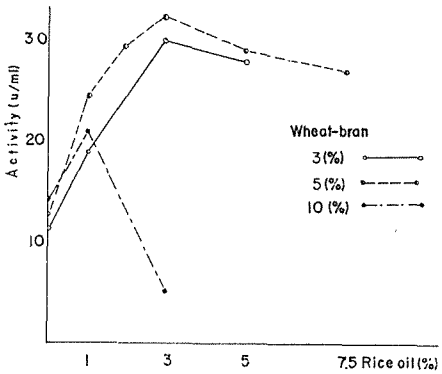

Fig. 6. Effect of rice oil on the enzyme prodction.
Each flask contained soluble starch 1.0%, KH_2PO_4 0.1%. Cultural period was twelve or sixteen days.

microorganism, and the enzyme production decreased. The relation between variation of rice oil and wheat-bran concentration for the enzyme production is shown in Fig. 6.

The activity in rice oil deficient medium showed little difference but it was increased by addition of rice oil as well as defatted-soybean. The highest enzyme activity was obtained in medium composed of wheat-bran 5 %, rice oil 3 %, soluble starch 1 %, and potassium biphosphate 0.1 %. Rice oil added in excess, the enzyme production decreased. In the case of medium containing wheat-bran 10 % for example, the enzyme activity showed the maximum value at rice oil 1 %. This phenomenon was considered to occur owing to the lack of agitation effect of the broth by an increase of viscosity because of oevrgrowth of the microorganism. Even in medium containing rice oil 10 %, the growth of the microorganism take place normally, but the enzyme was not produced.

Yield of amylolytic enzyme was greatly increased by addition of rice oil in cultural medium. The experiments described below were initiated in order to determine whether or not the enzyme production was increased by addition of another vegetable,

Table 10. Effect of oils on the enzyme production.

Group 1	A.	Group 2	A.	Group 3	A.
camellia oil	30.8	soybean oil	24.0	chinese-tung oil	10.2
palm oil	30.5	olive oil	23.8	japan tallow	9.5
rice oil	30.1	cotton seed oil	22.4	castor oil	8.1
colza oil	28.7	perilla oil	18.9	paraffin	5.7
seasame oil	26.5	linseed oil	17.3	paraffin oil	0
peanut oil	26.0	whale oil	24.6	vaselin white	0
lard	26.6	cod-liver oil	21.1		
		beef-tallow	14.8	control	11.1

The enzyme activity (unit/ml) is marked A., The composition of medium was as follows : wheat-bran 3.0 %, soluble starch 1.0 %, KH_2PO_4 0.1 % and each oil 3.0 %. Cultural period was fourteen days.

animal, and mineral oils in medium. Effects of these oils on the enzyme production are shown in Table 10. When the oils of group 1 were used, the enzyme production was performed almost similarly compared with rice oil. The oils of group 2 were somewhat effective, but those of group 3 gave no or negative effect for the enzyme production. No two oils in each group have similar physical or chemical properties.

The amount of oil in the broth was measured during the culturing period to examine whether or not the enzyme production was activated by additional oils acting upon the microorganism only physically, or being assimilated by the microorganism.

Time course of culture in shaking flask is given in Fig. 7.

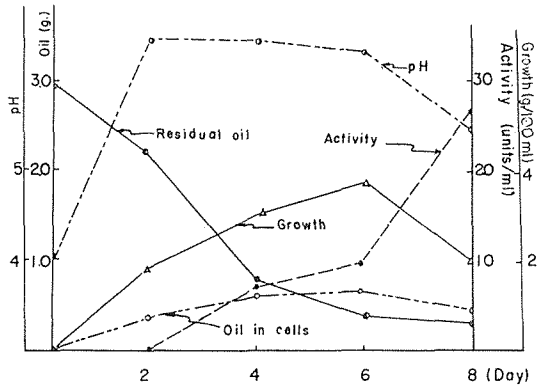

Fig. 7. Time course of the culture.

The oil in medium was almost assimilated in six days, and the oil in the microorganism showed the maximum value on the sixth day. The pH of the broth obtained its maximum value 5.5 on the second day, and remained constant until broth out. The growth of the microorganism showed the maximum value on the sixth day, and then it decreased because of autolysis of the microorganism.

The time course of culture in fermentor is given in Fig. 8. The residual oil in medium did not change until about sixteen hours and decreased rapidly during next fifteen hours. The growth of the microoganism showed the maximum value on the

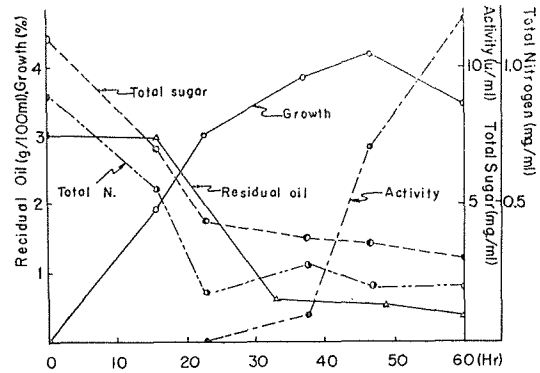

Fig. 8. Time course of the culture.

forty-fifth hours, and then it decreased due to autolysis as well as shaking culture. After twenty hours, total sugar and total nitrogen in the broth existed in very small quantities and then the enzyme began to be produced.

As it has been found that *Endomyces* sp. assimilated oil in medium, the present investigation was undertaken to confirm this phenomenon. The respiration of *Endomyces* sp., in the case of using rice oil as the substratum, was measured by the Warburg's manometric method.

Carbon dioxide evolution was oxygen uptake by *Endomyces* sp. are shown in Fig. 9.

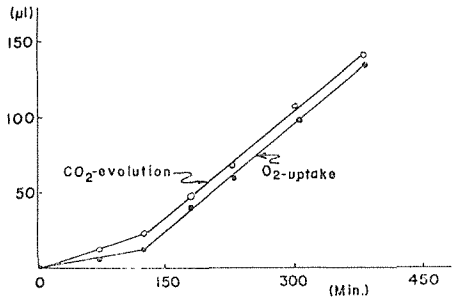

Fig. 9. Respiration of *Endomyces* sp.

Carbon dioxide evolution and oxygen uptake did not take place actively until one hundred and fifty minutes, but over this point the cells showed active respiration, and the respiration quotient was 1.32. It may be concluded from this results that *Endomyces* sp. assimilated rice oil.

4. Lipase

As the experiments described above prove that the microorganism assimilates the additional fat, this investigation is initiated in order to check on lipase of which fat could be hydrolyzed biochemically. Lipase activity of each of the media containing 1, 3, and 5 % of rice oil was determined. These results are shown in Fig. 10.

The depression and elevation of its activity with changes in the concentration of the additional oil were observed. The medium containing 3 % of oil showed the

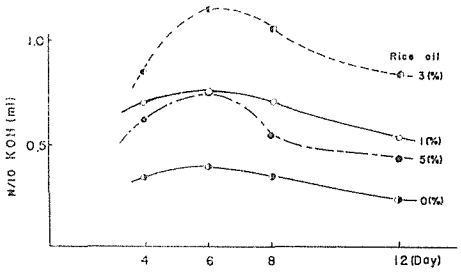

Fig. 10. Production of extracellular lipase. Each medium contained soluble starch 1.0%, bouillon 2.0%, and KH_2PO_4 0.1%.

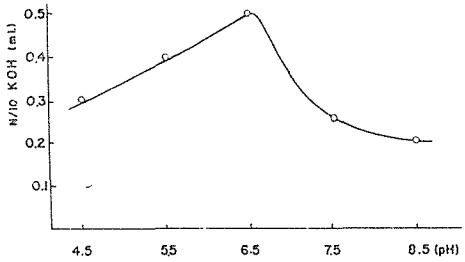

Fig. 11. Optimum pH of the lipase.

maximum activity. The lipase activity of broth started to rise within four days and reached a maximum in six days. K. Yamada[15] reported that *Candida cylinaracea nov* sp. produced an extracellular lipase which was proved to be useful. The lipase activity of *Endomyces* sp. was lower than C. *cylinaracea*.

The optimum pH of the lipase was determined under several pH of buffer solution and emulsions. This result is shown in Fig. 11.

It has been found that the optimum pH of th enzyme was about 6.5.

5. Intercellular Enzyme

This investigation was undertaken to determine the intercellular enzyme by the method described above. The intercellular lipase was not detected, but amylase was determined a little. The time course of the amylolytic enzyme production in cells is shown in Fig. 12.

From this result, it was found that the intercellular lipase did not exist and that the intercellular amylase remained a little, but most of the enzyme existed in the broth.

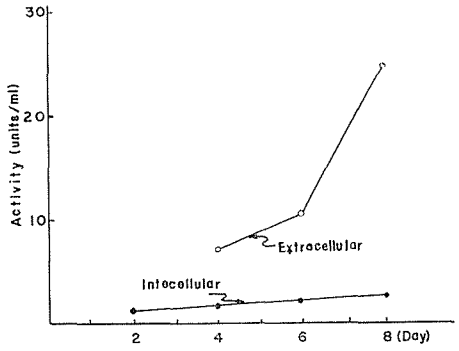

Fig. 12. Determination of intercellular amylase. The medium contained soluble starch 1.0%, bouillon 2.0%, rice oil 3.0%, and KH_2PO_4 0.1%.

6. The chemical Changes of Additional Fat during Culture

It has been described above that *Endomyces* sp. produced extracellular lipase. It was supposed naturally that the additional fat was hydrozed by lipase and free fatty acids were produced. This investigation was undertaken to determine the acid value and iodine number. The determination of the acid value was performed in order to examine the changes of free fatty acids in the extracted rice oil. The acid value was a measure of the amount of free fatty acids and was defined as the number of milligrams of potassium hydroxide required to neutralize the free fatty acids in 1 g. of sample. The determination of iodine number was performed in order to investigate the increase and decrease of double bond in fatty acids and fat during the fermentation. The iodine number was a measure of the unsaturation of the fat and was defined as the number of grams of iodine absorbed by 100 g. of the substance. The composition of fatty acids in rice oil is given in Table 11.

The results of this investigation are shown in Fig. 13 and 14.

Table 11. Fatty acids composition and analtical value of rice oil.

Saturated fatty acids			Unsayurated fatty acids			I. v.	A. v.
C_{14}	C_{16}	C_{18}	C_{18-1}	C_{18-2}	C_{18-3}		
0.4-1	13-18	1-3	40-50	29-42	0-1	106.5	0

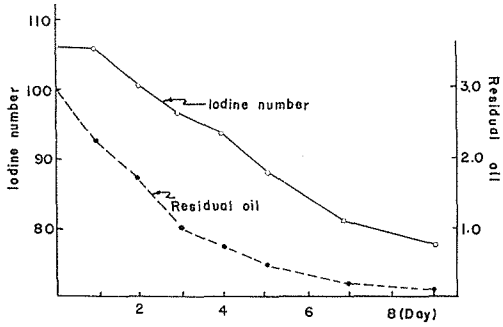

Fig. 13. Determination of iodine number. The medium contained soluble starch 1.0%, bouillon 2.0%, rice oil 3.0%, and KH_2PO_4 0.1%.

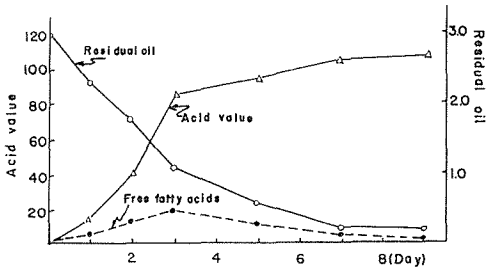

Fig. 14. Determination of acid value. The medium contained soluble starch 1.0%, bouillon 2.0%, rice oil 3.0%, and KH_2PO_4 0.1%.

The acid value of the recovered oil from the medium started to rise rapidly after inoculation and reached a value 88 in three days. Arriving at 88, the acid value increased gradually and reached 111 in nine days. It has been found from this data that the free fatty acids were produced in medium. The free fatty acids can be calculated as oleic acid using the following equation:

Free fatty acid (g.)=Residual oil (g.) × Acid value × 0.503/100

The amount of free fatty acids reached the maximum value 0.48 g. in three days, and then, it decreased and showed 0.11 g. in nine days. The percentage of free fatty acids in the recovered oil from the cultural broth remained nearly stationary from 44 to 56 per cent.

The iodine number of the oil recovered from oil-supplemented fermentations decreased during the run. The iodine number of the first sample was 107.1, whereas the added rice oil had an iodine number of 106.5. The later decrease in iodine number was very marked and reached 78.3 in eight days. This value was definitely lower than the iodine number 110.1 of the free fatty acids obtained from the rice oil. Evidently the unsaturated fatty acids were utilized somewhat more rapidly than the saturated acids.

7. The Effect of Fatty Acids

The present investigations were undertaken to determine the effect of mixed, solid, and liquid fatty acids which were obtained by saponification of rice oil, and the effect of several fatty acids which were obtained commercially for the amylase production.

For the separation of the component acids the rice oil was saponified with potassium hydroxide in alcohol solution. Unsaponifiable matter was removed by extraction of the soap solution with ether. The soap solution was acidified with mineral acid, and

the fatty acids were extracted. The separation of solid and liquid fatty acids from the mixed acids was worked up in the usual manner using lead soap in ethanol. Gusserow initiated this technique in 1829, and afterwards Varrentrap brought about some improvements. Gusserow and Varrentrap used ether as solvent, but, in 1921, Twitchell found that ethanol gave better results than ether. The solubility of several lead soaps of in fatty acids in ethanol is given Table 12[16].

Table 12. Solubility of lead soaps of fatty acids in alcohol.

Saturated fatty acids lead soap		Unsaturated fatty aids lead soap	
Caprylic	soluble	Myristoleic	very soluble
Capric	fairly soluble	Palmitoleic	freely soluble
Lauric	fairly soluble	Oleic	soluble
Myristic	distinctly soluble	Linoleic	freely soluble
Palmitic	almost insoluble	Linolenic	freely soluble
Stearic	almost insoluble	Gadoleic	sparing soluble
Arachidic	insoluble		

Table 13. Effect of several fatty acids on the enzyme production.

Fatty acids (g)		Time (day) 2	3	4	6	8
Mixed	2.0	2.0	11.0	14.0	20.1	22.8
	3.0	2.1	11.2	15.9	21.7	24.7
Solid	0.1	0.9	2.1	2.3	2.5	2.8
	0.5	1.6	4.4	7.2	8.9	10.0
	1.0	2.1	4.9	9.5	13.3	15.4
	1.5	2.5	5.4	12.6	16.6	19.3
	2.0	2.5	5.8	13.1	17.5	21.0
	3.0	2.3	5.3	11.9	14.5	18.9
Liquid	3.0	1.6	3.2	5.1	7.0	7.4
Palmitic	1.0		3.0	7.0	16.6	17.8
	2.0		1.2	3.0	8.8	10.7
Stearic	1.0		2.6	6.1	14.2	15.2
	2.0		0.9	2.3	5.4	7.0
Oleic	2.0		0.9	1.9	2.5	2.6
Rice oil	3.0		7.0	11.4	20.1	27.3

Each medium contained bouillon 2.0 %, soluble starch 1.0 % and KH_2PO_4 0.1 %.

The unsaponifiable matter, glycerol, and mixed fatty acids were separated from rice oil by saponification with potassium hydroxide. Unsaponifiable matter and glycerol were not effective on the amylase production. The results of this investigation are given in Table 13. The amylase activity of medium containing 3 % of mixed fatty acids reached 24.7 units in eight days and this value was slightly lower than the activity of the medium containing 3 % of rice oil. In view of the above fact, the effective matter in fat on the enzyme production was fatty acids. In an effort to determine the effect of the mixed fatty acids, it was separated to the solid and liquid fatty acids and they were added to the medium in order to determine their effect. The iodine number of the solid fatty acids was 39.0 and the number of the liquid fatty acids was

137.7 The amylase activity of the medium containing 2.0 % of solid fatty acids showed 21.0 units in eight days, and it was about seven-neinth of the activity of the medium containing rice oil. The amylase activity in the medium containing 3.0 % and 1.5 % of solid fatty acids was somewhat lower than the medium containing 2.0 % of the acids. The activity of the lipids deficient medum was 4.9 units in eight days. The addition of 0.1 % of solid fatty acids gave no improvement in the yield of the amylase. The medium containing 3.0 % of liquid fatty acids had little effect on the enzyme production but it allowed favorable growth of the microorganism. In view of the above facts, the reasonable conclusion is that the effective components of rice oil are solid fatty acids and that liquid acids have little effect on the enzyme production but they are effective to obtain large amount of microorganism. Saturated fatty acids, major part of solid fatty acids, were added into medium in order to determine the effect on the enzyme production. Saturated straight-chain fatty acids containing 2 to 10 carbon atomes inhibited the growth, and lauric and myristic acids allowed a little growth but gave no improvement in the yield of the enzyme. Palmitic and stearic acids were effective for the enzyme production and allowed normal growth. The amylase activity of medium containing 2 % of palmitic acid reached 10.7 units in eight days. Stearic acid gave good yield of the enzyme but it was less effective than palmitic acid. A few unsaturated acids containing 18 carbon atomes were tested, oleic, linoleic, and linolenic acids (one, two, and three double bond, respectively) were not able to improve the yield of the amylase and only oleic acid allowed normal growth of the microorganism.

8. Utilization of Fatty Acids

The data on effect of fatty acids on the enzyme production reported above indicate that the difference in the enzyme activity appeared according with the kind of fatty acids. This investigation was undertaken to determine the relation between the enzyme production and utilization of fatty adids. Mixed, solid, and liquid fatty acids obtained by saponification of rice oil, and palmitic, steraic, and oleic acids obtained commercially were used in these experiments. The amount of lipids consumed by the microorganism was determined by adding a weighed amount of lipid to medium.

Table 14. Utilization of fatty acids.

Lipids (g)		Time (day) 1	2	3	4	6	8
Mixed	2.0	0.50	0.89	1.30	1.62	1.78	1.79
	3.0	0.30	1.25	1.92	2.25	2.55	2.70
Solid	2.0	0.68	1.08	1.21	1.29	1.39	1.43
	3.0	0.74	1.46	1.72	1.92	1.98	2.04
Liquid	2.0	0.38	0.79	1.19	1.52	1.68	1.70
	3.0	0.56	1.12	1.74	2.18	2.35	2.39
Palmitic	2.0	0.35	0.60	0.75	0.82	0.90	1.00
Stearic	2.0	0.28	0.45	0.60	0.70	0.80	0.85
Oleic	2.0	0.37	0.75	1.12	1.44	1.46	1.50
Rice oil	3.0	0.40	0.83	1.65	2.10	2.70	2.80

Each medium contained bouillon 2.0 %, soulbe starch 1.0 % and KH_2PO_4 0.1 %.

The residual lipids were extracted with ether. The utilization of fatty acids is set out below in Table 14.

The assimilation of rice oil is added in the Table to compare with fatty acids. It has been found that mixed fatty acid was utilized almost similar to rice oil, and that the percentage of utilization was about 90 % in eight days. When 2 % of the lipid was added, mixed, liquid, oleic, solid, palmitic, and stearic acids were utilized 89.5, 85.0, 75.0, 71.5, 50.0, and 42.5 % respectively. On the basis of these data, it seemed reasonable to assume that the added unsaturated fatty acids was almost utilized, but they gave no effect on the enzyme production, and that utilization rate of the saturated fatty acids was lower than that of unsaturated acid. It may be considered that the differences of the rate of utilizing saturated fatty acids appeared to effect the yield of the amylase.

SUMMARY

During the course of an investigation of amylase production using *Endomyes* sp. it is desirable to use medium containing rice-bran. In an effort to determine the effect of rice-bran, it has been found that rice oil gives the improvement in the yield of the enzyme. From the results of investigation to determine the effect of some animal, vegetable, and mineral oil, it has been found that camellia, rice, and colza oil were very effective for the enzyme production. The additional rice oil in medium was utilized about 93% in eight days by *Endomyces* sp.. Extracellular lipase was detected a little and its optimum pH was about 6.5. It has been found that the effective component of rice oil is fatty acids and saturated acids are more effective for the enzyme production. From the results of determination of iodine number, the unsaturated fatty acids disappeared from the medium at a faster rate than the saturated acids, but unsaturated acids gave no improvement in the yield of the enzyme. The free fatty acids in medium started to rise in three days, and then, they decreased. Palmitic acid obtained commercially was more effective on the enzyme production than stearic acid, but the activity increased by the addition of palmitic acid was about a half of that increased by the addition of mixed fatty acids obtained by saponification of rice oil. The reason of this difference of the effect on the enzyme production may be considered due to the difference of utilization rate of fatty acids.

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