KOR. JOUR. MICROBIOL. Vol. 11, 121~128 (1973)

*Rhizopus*의 아밀라제에 관한 연구*(第三報)
——*R. niveus*의 생장생리——

李 永 祿 · 李 平 佑
(高麗大學校 大學院 生物學科)

Studies on the Amylase of *Rhizopus**(III)
——Nutritional and Cultural Characteristics of *R. niveus*——

LEE, Yung Nok, and Pyung Woo LEE
(Department of Biology, Korea University)

ABSTRACT

In order to clarify the best cultural conditions of *Rhizopus niveus* the effects of aeration, *p*H, and various nutrients, such as different carbon and nitrogen sources, vitamins, and growth substances, on the mycelial growth were studied through liquid culture, and amylase activities of the fungus at different cultural periods were measured.

Soluble starch, xylose and galactose are excellent sources of carbon for growth of the fungus. Sorbose and lactose are utilized slightly for growth. Peptone, ammonium sulfate and alanine are excellent nitrogen sources for growth, tryptophane and potassium nitrate are utilized slightly for growth and sodium nitrite is not utilized. Thiamine and gibberellin are excellent growth substances for the fungal growth, and biotin, nicotinamide and indole acetic acid (IAA) are also effective.

Rhizopus niveus grows better at rotatory culture than at stationary culture and earlier growth of the fungus increases remarkably at rotatory culture. Optimum *p*H range for growth of *Rhizopus niveus* is 4—5, and the fungus grows better at *p*H 6 than at *p*H 3. Growth increases linearly with an increase of soluble starch content up to 100g per liter medium, but 5 grams of ammonium sulfate per liter is the optimum nitrogen concentration for growth, if Pfeffer's medium is employed.

Amylase activities of *Rhizopus* at different cultural periods showed that the maximum amylase production takes place after the cell population has reached its peak in the culture. Dextrinogenic amylase production has reached maximum at stationary phase, and maximum saccharogenic amylase production takes place in the phase of negative growth acceleration.

INTRODUCTION

Althouth the growth of the fungi different from genus *Rhizopus* have been studied extensively (Coley-Smith, 1960, 1966; Mchan and Johnson, 1970) no recent studies are found in the literature on the nutrition and the effect of specific nutrients of the fungus. Recently, Papavizas(1970) have reported some

* This work was supported by a grant from The Yonam Foundation

environmental conditions favorable for growth and relative efficiency of various carbon and nitrogen sources under the different cultural conditions on *Sclerotium*, and Nolan(1970) have studied the vitamin requirement of *Catenaria*. Although Yamazaki (1931, 1934), Yamamoto(1930), Takeda(1935) and Inui *et al.*(1964) have reported some physiological characteristics on *Rhizopus* species for taxonomical study, no comprehensive nutritional and physiological studies of the fungus have yet been made.

In the present study, the effects of various nutrients such as different carbon and nitrogen sources, vitamins, and growth substances, on the mycelial growth were measured through liquid culture in order to clarify the best nutritional conditions of the fungus, and amylase activities of the fungus at different cultural periods were measured.

MATERIALS AND METHODS

1. Origin and maintenance of the strain.

The strain of *R.niveus* IAM 6035 was obtained from The Institute of Applied Microbiology, University of Tokyo. Stock culture were maintained on slants of a potato starch medium and were transfered at 2 to 4 week intervals. Stock cultures were incubated at 17±1°C.

2. Inoculum preparation and culture media

The method for obtaining a spore inoculum was a modification of the technique used by Carlile and Machlis(1965). 2 Loopfuls of seven-days-old strain were harvested with 30m*l* sterile distilled water to yield a spore suspension, and 1 m*l* portions of which were added to the experimental flasks. Two basal media and Pfeffer's solution were used throughout. Basal medium 1 (BM—1) was the same with Pfeffer's medium without sucrose. The second basal medium(BM—2) was the same with Pfeffer's medium without ammonium nitrate. Experimental media were adjusted to *p*H 5.0 before autoclaving.

3. Growth studies

Experimental cultures were grown in 250m*l* Erlenmyer flasks containing 50m*l* of medium. All cultures were incubated stationary at 30°C, but rotatory cultures were incubated on a horizontal shaker.

Four groups of carbon sources were studied; 7 monosaccharides (2 pentose and 5 hexoses), 3 disaccharides, 1 trisaccharide, and 4 polysaccharides. All carbon sources were added to BM—1 at the ratio of 5gr carbon *per* liter.

Three groups of nitrogen sources were studied; 7 amino acids, 6 inorganic salts, and 4 complex organic sources of nitrogen. The nitrogen sources were added to BM—2 at the ratio of 0.5gr nitrogen per liter.

Seven growth substances were studied; vitamins and growth substances were added to Pfeffer's media. The concentration of thiamine was 150μg per liter while that of the other vitamins were 100μg per liter and that of growth substances were 150 ppm.

Mycelium on the synthetic media containing single carbon or nitrogen sources was assayed after 2 and 4 days of inoculation.

Optimum *p*H study was carried out in Pfeffer's solution adjusted from *p*H 1 to 9 and comparative growth experiments between rotatory and stationary culture were carried out in Pfeffer's so-

lution as well as in the case of optimum *p*H study. Mycelium was harvested at predetermined intervals.

When the mycelium from liquid media was harvested, it was collected on a filter paper, washed with distilled water, and dried overnight at 100°C before being weighed. All dry weights were rounded off to the nearest milligram.

4. Preparation of enzyme solution

Culture medium used in this experiment was Pfeffer's solution. Fifty milliliters of culture media were inoculated and incubated at 30°C in 250m*l* flasks. Mycelium harvested at predetermined intervals was dried and the supernatant was given to enzyme solution.

5. Determination of amylase activities

Amylase activities were measured by colorimetric method.

1) Dextrinogenic amylase

1M*l* enzyme solution was added to an equivolume of acetate buffer (*p*H 5.0) and incubated for 10 minutes at 37°C. After incubation 2m*l* of 2% soluble starch solution was added to it, and then the mixture was further incubated for 30 minutes. For determination of dextrinogenic activities, the reaction mixture was added to 5m*l* of 1N acetic acid and then was diluted with 50m*l* distilled water, added to 5m*l* of 0.1N I_2-KI solution. After all additions have been made, the optical density was mesuared at 700mμ and dextrinogenic amylase activity was represented to RDP (relative dextrinizing power) as in the previous paper (Lee and Yoon, 1973).

2) Saccharogenic amylase

Determination of reducing sugar as glucose was carried out with the same method as in the previous paper (Lee and Yoon, 1973).

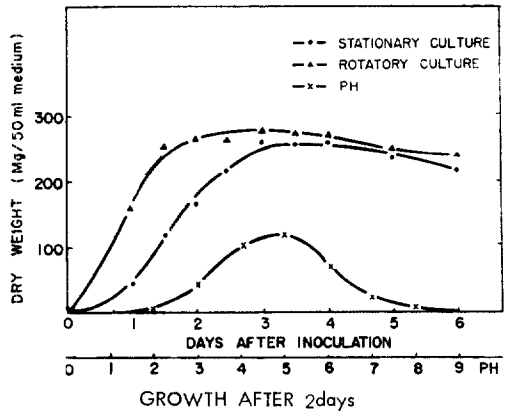

Fig. 1. The comparison of mycelial growth of *R. niveus* in rotatory and stationary culture, and the optimal *p*H range for growth of the fungus. Mycelium was harvested after 2 days incubation.

RESULTS AND DISCUSSION

1. Effect of aeration on mycelial growth

To determine the effect of aeration, one was shaked and the other was stayed at 30°C. Pfeffer's solution was incubated with the inoculum and harvested after predetermined intervals. Mycelia were grown at submerged state in shaked culture and on surface in stationary culture. Growth rate was significantly higher in submerged culture than in surface culture on the same medium, but total growth was not so much different. The results obtained are represented in Fig. 1. These results are similar to the results of Mchan and Johnson (1970) on *Monascus purpureus*.

2. Effect of *p*H for growth in liquid cultures

Mycelia in the medium adjusted to each *p*H were harvested after 2 days incubation, and then dried and weighed. The results, after 2 days of incubation, show that maximum growth was obtained at *p*H 5 and the strain grew

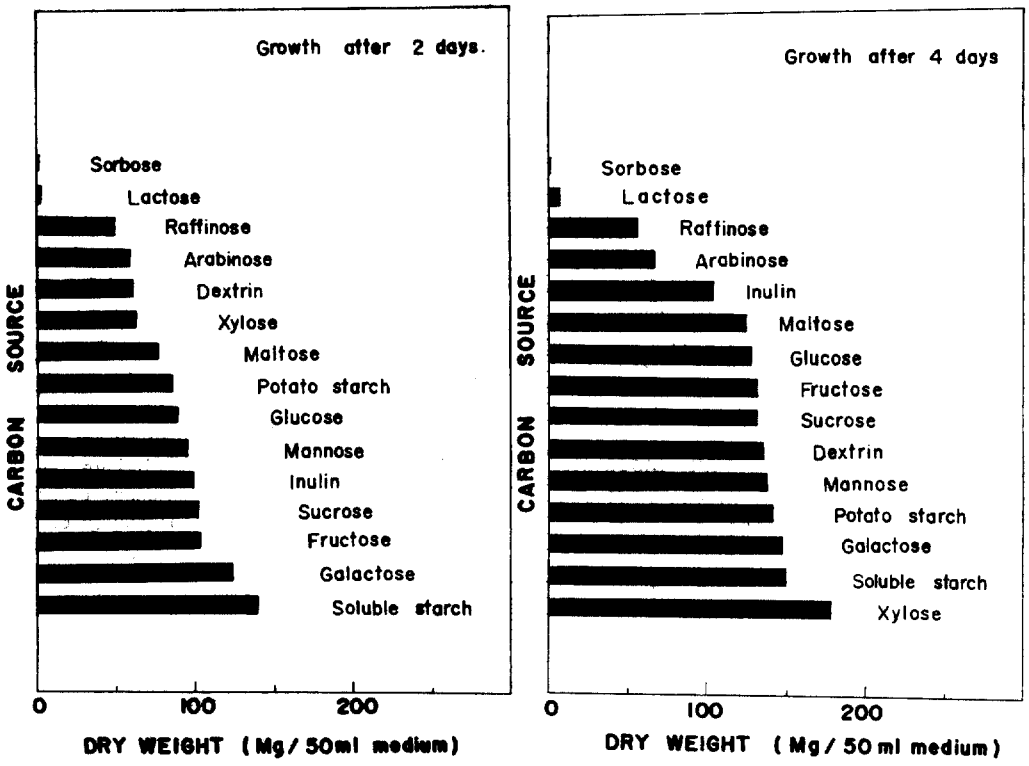

Fig. 2. Growth rate of *R. niveus* in basal medium 1 (BM—1) supplemented with carbon sources.

better at *p*H 6 than at *p*H 3. Growth failed at *p*H 1, 2 and 9, as shown in Fig. 1.

Yamamoto (1930) reported that the optimum *p*H for growth of *R. acidus* was *p*H 4.0 while no growth in the *p*H range of 1.8—2.35.

By using bouillon containing 5% glucose, Takeda(1935) found that *Rhizopus* species did not grow at *p*H 2.0 and some species formed only poor submerged mycelia at *p*H 2.2 after 7 day incubation periods, and that the optimum *p*H for growth was generally in the range of *p*H 4—5, and most species grew slightly at *p*H 6 and 9.

3. Carbon utilization for growth

Of 11 monosaccharides and oligosaccharides tested the best ones for growth

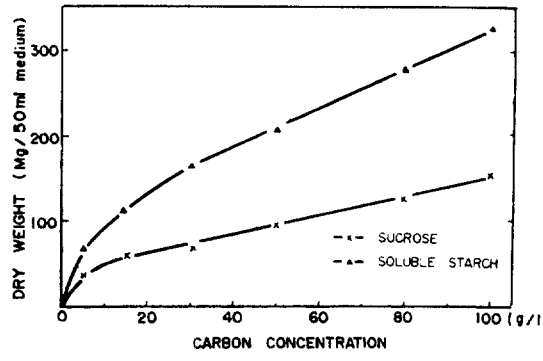

Fig. 3. Growth rate of *R. niveus* for 10 days in different amounts of soluble starch and sucrose.

were sucrose, maltose, glucose, fructose, mannose, galactose, and xylose. Especially xylose and galactose were good carbon sources for growth. Sorbose was utilized slightly and lactose supported some growth. All polysaccharides tested

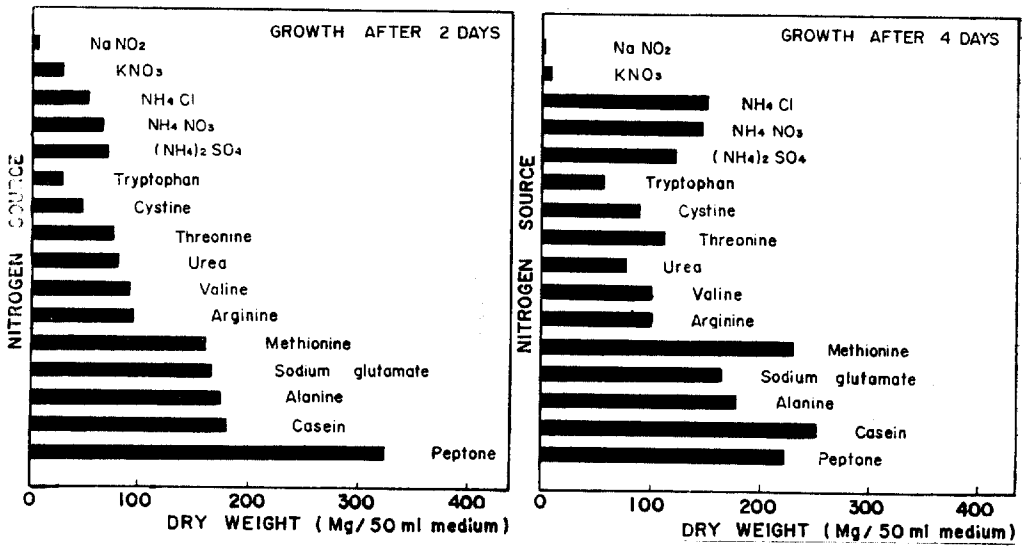

Fig. 4. Growth of *R. niveus* in basal medium 2 (BM—2) supplemented with different nitrogen sources.

supported good growth. Soluble starch was excellent carbon source for growth of the strain. The results after 2 and 4 days of incubation were shown in Fig. 2.

Effect of increasing concentration of soluble starch on mycelial growth is shown in Fig. 4. The possibility of improved growth of *Rhizopus* in BM—1 by increasing soluble starch concentrations is investigated with the strain in an experiment involving 6 soluble starch levels. As shown in Fig. 3, dry weight of the fungus after 10 days of growth was increased along the increase in starch concentration up to 100 gr per liter of medium.

4. Nitrogen utilization for growth

The best growth of the fungus was obtained with peptone, casein and sodium glutamate. Good growth was also obtained with ammonium sulfate, but ammonium chloride, ammonium nitrate and potassium nitrate were utilized slightly for growth and sodium nitrite was not utilized. The amino acid alanine and methionine were good sources of nitrogen. Valine, threonine, arginine and cysteine were relatively good nitrogen sources, but tryptophan was utilized slightly.

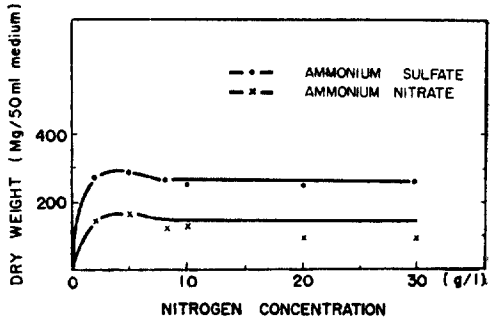

Fig. 5. Growth rate of *R. niveus* for 10 days in different amounts of ammonium sulfate and ammonium nitrate.

The results after 2 days incubation are represented in Fig. 4.

Ammonium sulfate and ammonium nitrate were selected for further studies

because these are best inorganic nitrogen sources for growth of *R. niveus*. To determine the optimum amount of nitrogen needed for growth, these nitrogen sources were added separately to BM—2 in different concentrations. As shown in Fig. 5, dry weight of the fungus is increased as the concentrations of nitrogen increase up to 5.0gr/liter, but growth with 10, 20, 30gr/liter was not good.

Yamazaki(1931) reported that fructose was the best carbon source for the growth of *Rhizopus* species. Takeda(1935) found that lactose was not assimilated, or only very poorly assimilated to form submerged mycelia by a few species. From a comparative study on the effects of various nitrogen compounds, Yamamoto(1930) reported that peptone was generally most suitable for growth of *Rhizopus* species. These reports are concordant with the results of the present studies.

5. Effect of growth substances on growth

To determine the effect of growth substances for growth, growth substances were added seperately to Pfeffer's solution.

As shown in Fig. 6, addition effect of gibberellin or thiamine was excellent for growth of the fungus. Thiamine was not effective than other growth substances for 2 days growth after incubation, but it was the most effective for 4 days growth after incubation. The analysis indicated that the yield for growth of the fungus with supplements of the various substances was not significantly different, and the fungus did not have an absolute requirement for an external vitamin sources.

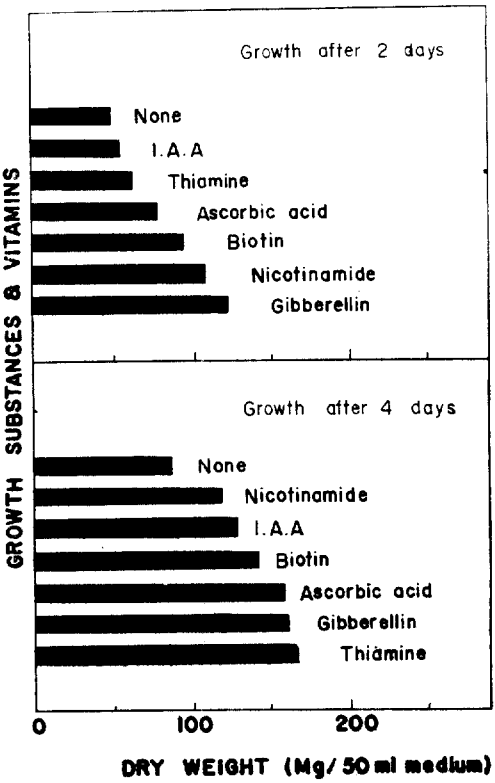

Fig. 6. Growth rate of *Rhizopus niveus* in Pfeffer's medium supplemented with various vitamins and growth substances.

6. Variation of amylase production during the growth periods

Amylase activities of *Rhizopus* at different cultural periods showed that the maximum amylase production took place after the cell population had reached its peak in the culture. Dextrinogenic amylase production of *R. niveus* had reached maximum at stationary phase, and maximum saccharogenic amylase production took place in the phase of negative growth acceleration.

The results obtained are represented in Fig. 7. These results are concordant with the report of Nomura *et al.*(1956) and Fukumoto *et al.*(1958) with the bacterial culture they employed.

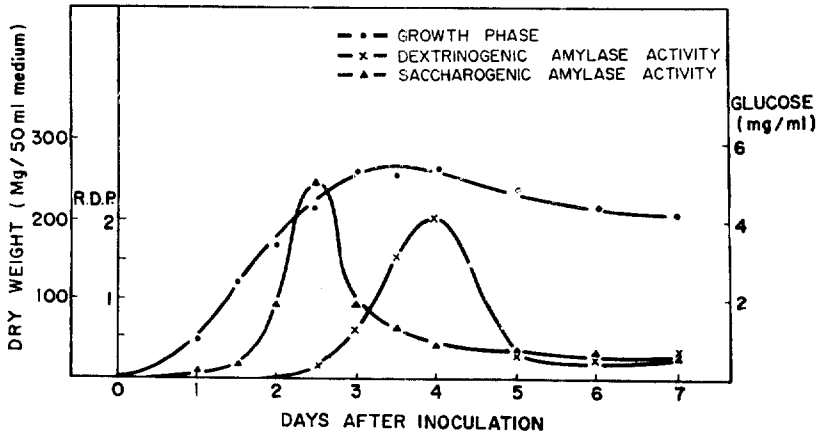

Fig. 7. Changes in amylase activities of *Rhizopus niveus* during the growth phase.

摘 要

*R. niveus*의 최적배양 조건을 밝히고저 菌體의 生育에 미치는 진탕효과, *p*H 및 여러가지 영양소의 영향을 측정하고 배양시기에 따른 菌體의 아밀라아제 생성능을 조사하였다.

가용성 전분, 크실로오스 및 가락토오스 등은 균사의 생장에 가장 효과적인 탄소원이었으나 소르보오스 및 락토오스는 거의 이용되지 아니하였다. 페프톤, 황산암모니움 및 알라닌은 균사의 생육에 가장 효과적인 질소원이었으나 트리프토판과 질산염은 거의 이용되지 아니하였고 아질산염은 전혀 이용되지 아니하였다. 공시한 모든 생장소가 효과적이었으나 그 중에서도 티아민과 지베렐린은 특히 균사의 생장을 촉진시켰다.

*Rhizopus*는 정치배양 보다는 진탕배양에서 잘 자랐고 특히 초기 생장이 진탕배양에서 증가하였다. 生育을 위한 최적 *p*H는 4~5였으며 *p*H 2이하에서나 *p*H 9 이상에서는 거의 자라지 아니하였다. Pfeffer's medium을 사용하였을 때 炭素源의 농도가 배지 *l*당 100gr에 이르기까지 생장이 증가 하였으나 질소원의 경우에는 배지 *l*당 5gr이상에서는 증가가 수반되지 아니하였다.

배양시기를 달리하는 *Rhizopus*의 아밀라아제 생성능은 菌體의 生長이 極大에 달한 후에 극대에 이른다는 것을 나타내었다. 덱스트리노제닉 아밀라아제 생성은 정지기에 극대에 도달하였고, 사카로제닉 아밀라아제 생성은 역생장촉진기에 극대에 도달하였다.

REFERENCES

1. Carlile, M.J., & Machlis, L., 1965. The response of male gametes of *Allomyces* to the sexual hormone sirenin. *Amer. J. Bot.*, **52**, 478—483.
2. Coley-Smith, J.R., 1960. Studies of the biology of *Sclerotium cepivorum* Berk. 4. Germination of sclerotia. *Ann. Appl. Biol.*, **48**, 8—18.
3. Coley-Smith, J.R., and R.W. Holt, 1966. The effect of species of *Allium* on germination in soil of sclerotia of *Sclerotium cepivorum* Berk. *Ann. Appl. Biol.*, **58**, 273—278.
4. Fukumoto, J., Yamamoto, T., Tsuru, D., and Ichikawa, K., 1958. Proc. Intern. Symp. 1957. Enzyme Chem., Tokyo, Kyoto, 479—482.
5. Inui. T., Y. Takeda, and Iizuka, H., 1964. Taxonomical studies on genus *Rhizopus*. *J. Gen. Appl. Microbiol.*, II, Suppl. 1—121.
6. Lee, Y.N., and K.H. Yoon, 1973. Studies on the amylase of *Rhizopus*(Ⅱ). *Kor. Jour.*

Microbiol., **11**, 89—100.

7. Mchan, F., and G.T. Johnson, 1970. Zinc and amino acids; Important components of a medium promoting growth of *Monascus purpurcus*. *Mycologia*, **62**, 1018—1030.
8. Nolan, R.A., 1970. Sulfur source and vitamin requirements of the aquatic Phycomycetes, *Catenaria anguillulae*, *Mycologia*. **62**, 568—577.
9. Nomura, M., Maruo, B., and Akaboris, S., 1956. *J. Biochem.*, Tokyo, **43**, 143 in "*Advances in Applied Microbiology*" (Umbreit, W.,W. ed., **7**, 287)
10. Papavizas, G.G., 1970. Carbon and nitrogen nutrition of *Sclerotium cepivorum*. *Mycologia*, **62**, 1195—1203.
11. Takeda, Y., 1935. Report No. 147 of the Dept. Industry, Gov. Research Inst. Formosa.
12. Yamamoto, Y., 1930. Ein Beitrag zur Kenntnis der Gattung *Rhizopus*. *J. Facult. Agr.*, Hokkaido Imp. Univ., **28**, pt. 1, 68—69.
13. Yamazaki, M., 1931. Jozogakaku Zasshi. *J. Brewing Japan*, **9**, 551.
14. Yamazaki, M., 1934. On the classification of *Rhizopus* species. *Bull. Utsunomiya Agr. Coll.*, **5**, 1—16.