KOR. JOUR. MICROBIOL Vol. 15, 63~76 (1977)

〈Article〉

Amylase Production by Continuous Cultures of *Aspergillus oryzae* and its Mutants

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*Aspergllus oryzae*와 그 變異株의 連續培養에 의한 Amylase 生成에 관한 硏究

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ABSTRACT

Irradiation with high doses of gamma rays induced the reduction of mycelial weight and amylase activity, and increased relative amylase activity in surface cultures. Biphase in growth curves was shown in aeration-agitation cultures but the behavior of the first phase of growth could be eliminated by replacing the amylasehydrolysed starch substrates, so that enzyme production was shortened *ca.* 40 hours and relative amylase activity was increased about 3 times higher before onset of autolysis.

In the effect of gibberellin on amylase production, the positive stimulation was appeared to only surface cultures of the liquid medium and the negative effect to shake-cultures in a mutant.

Trials of various continuous culture were resulted not only the approach to the value of amylase activity in surface cultures of liquid medium, but also higher productivity than in batch cultures.

The culture-degeneration was observed in two-stage continuous culture, but did not appear in continuous elevation culture.

INTRODUCTION

The production and application of microbial amylase have been reviewed in many literatures(Meyrath, 1957; Windish *et al.*, 1965; Banks *et al.*, 1967). Amylase production by *Aspergillus oryzae* has been done industrially with wheat bran and vermiculite supplement as surface cultures of solid medium.

In the industrial point of view, these methods have disadvantages since lots of labour hour and space are necessiated, and mechanization and purification are difficult (Meyrath *et al.*, 1971).

There is a certain trend to apply submerged culture and surface liquid culture for the production of enzyme by moulds (Meyrath, 1965).

A new device was designed which is

suitable for laboratory and industrial scale cultivation of microorganisms, particularly fungi, as the surface culture of liquid medium(Kybad *et al.*,1976). It does not use a high consumption of energy for stirring and aeration, and does not require maintenance of complicated equipment.

Mycelia of cellulolytic basidomycete *Phanerochaete chrysogenum* has been grown in submerged cultures to harvest the product attractive as potential food ingredient(Hofsten *et al.*, 1975).

The continuous cultivation with filamentous microorganisms can be found in the performance to obtain such products as penicillin by *Pen. chrysogenum*(Pirt *et al.*, 1960), novobiocin by *Strep. niveus*, streptozoacin by *Strep. achromogenes* and steroid bioconversion (pregnatriene) by *Streptomyxa affinis*(Means *et al.*, 1962).

Amylase production of *Aspergillus oryzae* has been seldom found in case of the continuous culture(Braun, 1972).

Continuous cultures and submerged cultures have been not preferably used in fungal amylase production, since the yield of amylase is less than a half in surface cultures of solid or liquid media(Meyrath *et al.*, 1971). However, it is reported that yields of dextrogenic amylase by *B. subtilis* strain NRRL B-941 appeared to be as high by submerged culture as by surface culture (Dunn *et al.*, 1959).

Thus this study deals with methodological problems in fungal amylase production whether or not semicontinuous culture and continuous culture are able to employ as done in other microorganisms, and does the induction of mutants as circumvened advantages of both rapid initial development and higher specific amylase production.

MATERIALS AND METHODS

1. Microorganism

Aspergillus oryzae (Ahlburg) Cohn was used in these experiments, which has been used in The Institute of Applied Microbiology in Vienna.

2. Gibberellin

Pure gibberellin was used (Fluka-AG Chemical Factory, Nr. 593588).

3. Media

Sporulation medium contained per litre of distilled water; starch, 40g; $(NH_4)_2SO_4$, 8g; $MgSO_4 \cdot 7H_2O$, 0.3g; $FeCl_3 \cdot 6H_2O$, 0.003g; KH_2PO_4, 34g; Na_2HPO_4, 35.5g; agar, 20g. For sporulation it was distributed 150 ml amounts in one litre Roux flask (tissue culture flask) horizontally and autoclaved for 15 mins. at 121°C. Then it was allowed to solidify at room temperature. *p*H was adjusted to 6.8 by itself.

Production medium contained per litre of distilled water; starch, 40g; $(NH_4)_2SO_4$, 16g; $MgSO_4 \cdot 7H_2O$, 0.6g; $FeCl_3 \cdot 6H_2O$, 0.1g; KH_2PO_4, 4g; citric acid, 10g; acetic acid, 6.9ml; yeast extract, 2g. *p*H was adjusted to 6.8 with 20% NaOH solution. For the plates 20g agar was added to the above mentioned medium. Sterilization was done for 15 mins. at 121°C.

4. Preparation of conidia suspensions

The conidia were incubated on sporulation medium for 7 days at 30°C. The conidia were harvested by aspiration apparatus (Witt, 1968), and suspended them into 100ml sterile water amounts in 250ml Erlenmeyer flasks. Thereafter, suspensions were shaken to sufficiently segregate clumps of conidia into individual conidium on the orbital shake-incubator. After filtering through sterile cotton, conidia were counted with Haemocytometer. Inocu-

lum size in all experiments was 10^6 conidia/10ml medium.

5. Preparation of hydrolysed starch

In preparation of amylase, 1.5 litre acetone (96%) was added to 1 litre fitrate obtained from cultures of a mutant Nr. 518 of which activity was 200 units per ml and *p*H was 7.8. It was kept at 4°C over night.

Precipitates were harvested by carefully decanting supernatant and dried in a vaccum desicator at room temperature for a day.

About 20g crude amylase powder was recovered. The amylase activity was 10,000 units/ml. 10g amylase was added to 2 litre production medium containing 120g soluble starch. *p*H was adjusted to 5.5 with 20% NaOH solution. Hydrolysis was done at 40°C for 35 mins. until the colour reaction with iodine was disappeared, then total volume was made to 3.0 litre with distilled water. *p*H was readjusted to 6.8 with the same alkaline solution.

Immediately the medium was autoclaved at 121°C, for 30 min to inactivate the rest enzyme activity.

6. Irradiation

Irradiation was carried out in a cobalt-60 irradiator. Dosimetry showed that the cell produced 0.43 mrad per an hour. 10 ml suspensions containing 3×10^7 conidia/ml of distilled water was irradiated in screw-capped bottles of 20 ml volume. While irradiated, bottles were immersed in ice. Two doses of 120 krad and 150 krad were chosen to produce more than a 99.99 % mortality (Porshnyykova *et al.*, 1970; Meyrath *et al.*, 1971).

7. Screening techniques

Agar was melted to the production medium for screening techniques. While in a hot state, 30 ml medium was pippeted and poured into plastic Petri dishes to give an even surface. It was allowed to solidify at room temperature. They were kept at 4°C for 4 days in the refrigerator, so that an opaque medium was obtained from the retrogradation (reprecipitates) of starch. This opaque medium was suitable for detecting the clearing zone obtained by amylolytic activity.

On the prepared Petri dishes, 0.2 ml diluted suspensoin was plated out by spreading with flame sterilized L shape glass rod. They were then incubated at 30°C. After 4 days incubation, prospective colonies were picked up according to the ratio of diameter of colony to clearing zone, and transferred to slants of the sporulation medium.

8. Gibberellin tests

Gibberellin was added to 10ml production medium to be the concentration of 5 ppm to 150 ppm. Media were sterilized at 121°C for 20 minutes and cooled at room temperature.

Conidia were inoculated to contain 10^6 conidia per 10 ml production medium in 100 ml Erlenmeyer flasks, and these were incubated at 30°C for 4 days.

The wild strain and a mutant Nr. 518 were cultivated in stationary liquid cultures and shaken cultures (250 rpm) in the orbital incubator.

9. Estimation of mycelial dry weight

The weight of mycelium in 10ml fermented medium was estimated by collecting it on nylon filter in a Buchner funnel. After washing with distilled water and pressing alternatively with rubber bung, pellets were made, and dried at 105°C over night.

Mycelial dry weight was designated as

MDW mg/10 ml fermented medium.

10. Determination of amylase activity

Test tubes containing 0.7 ml starch solution (0.1% solution buffered with M-sodium citrate) were placed in a water bath at 20°C, adding 0.5 ml portion of the suitable diluted enzyme solution.

The reaction was stoppped at 2 and 4 minutes by adding 10 ml test solution of iodine (ca. 7×10^{-4}N). The decrease of colour density was recorded by EEL photometer using 2 cm cuvettes and neutral filter. Units were calculated by the following formular:

$$E=\frac{100^{+}-X}{T\times100}\text{(unit/ml)}$$

where E is amylase unit per ml filtrate, 100^{+} is the optical density of the blank, X is optical density of sample after time T in minute and 100 is division factor to obtain reasonably small figure as activity.

The value of X should be within the range of 50 to 90 optical density against the blank value (Meyrath, 1965).

11. Stationary liquid culture

500 ml production medium was autoclaved at 121°C for 15 min and 5 ml conidial suspension containing 10^{7} conidia / ml was added aseptically into this medium and mixed thoroughly. Then 10 ml medium was pippetted and transferred into 100 ml Erlenmeyer flask, which were wrapped with cut-pieces of Fimulin Watte (baby napkin) and were closed tightly with gum rings and sterilized at 160°C for 2 hours. These flasks were incubated at 30°C for 7 or 8 days.

12. Submerged culture

Submerged culture was performed in 5 litre Vogelbusch fermenter (Austria). Air was supplied through the glass tube packed with cotton wool by Hyflo air pump.

Constant temperature in the fermenter was regulated by Tempunit (England). Submerged culture in nonhydrolysed starch and hydrolysed starch substrate was carried out with mutants under such condition as inoculum size was 10^{6} conidia/ml, air was inleted at the rate of 900ml/min/*l* of substrate, medium was agitated at speed of 1500 to 2000 rpm and 1ml Glanapon as antifoam was added per litre medium.

Culture volume was 3 litre in hydrolysed starch substrate and 3.5 litre in nonhydrolysed one.

13. Two-stage continuous culture

Two-stage continuous cultures were performed with the mutant Nr. 518 at 30°C. In the first vessel two litre production medium was hydrolysed by amylase for only batch culture. Sterile air was introduced into the fermenter at a rate of 100 to 225ml/min/L substrate.

Agitation was at speed of 1000 to 2000 rpm and regulated to maintain the even surface of culture. Other conditions were the same as in 12. In the second vessel two-stage fermentation was fulfilled as connection with the first vessel. The flow was continued until two litre of fermented medium was allowed to reservoir in the second vessel and then meduim was flowed out.

Fermentation conditions were maintained at a rate of 100ml/min in aeration and at a rate of 75ml/hr in addition of medium. Only the agitation was kept altered at speed of 700 rpm.

14. Continuous elevation culture

The continuous elevation culture was

performed with the wild strain at 30°C by continuous repetition of both the elevation where culture liquid is added a to certain level of volume in the fermenter and the removal where fermented liquid is withdrawn to original volume in a given time.

Culture volume was 3 litre. The inoculum size was 10^6 conidia/10ml. Cotton filtered air was inleted at a rate of 100ml/min/*l* of substrate. Agitation was occasionally regulated within a range 550 to 2,400 rpm in order to protect from the formation of packed mycelium.

After accomplished batch cultures, two litre fermented medium was removed from the fermenter. The fresh medium was supplied for 24 hours at rate of 83.3ml/min and two litre fermented medium was taken out everyday.

Samples were checked every 4 hours.

RESULTS

1. Surface cultures of mutants

According to screening techniques, 11 promising mutants could be selected of higher ratio than that in the wild strain. isolated mutants were incubated for 8 days as stationary liquid cultures.

The results are shown in Table 1. Mutants showed the reduction of mycelial dry weight and amylase activity, and higher relative amylase activity in mutants Nr. 212, Nr. 514 and Nr. 518.

2. Growth in nonhydrolysed starch substrate

Submerged cultures were carried out with finally selected mutants. Fig. 1 shows the development of culture in nonhydrolysed starch substrate.

Mutants Nr. 212, Nr. 514 and Nr. showed two phase of growth (bigrowth) in 96 hours. The first maximal growth reached 80mg MDW/10ml and 58mg MDW/10 ml after 24 hours in mutants Nr. 514 and Nr. 518 respectively, and 74mg MDW /10ml after 36 hours in a mutant Nr. 212.

Then growth was declined slowly until the second growth was restarted. The second maximal growth reached 183mg MDW/10ml and 147mg MDW/10ml after 84 hours in mutants Nr. 514 and Nr. 518 respectively, and 163mg MDW/10ml after 96 hours in mutant Nr. 212.

Amylase activity in the first phase was lower but in the second phase increased in nearly parallel with mycelial increase. It reached 108 units/ml at 96 hours in a mutant Nr.212, and 130 units/ml at 84 hours in mutants Nr.514 and Nr. 518.

On the contrary amylase activity in the wild strain was increased rapidly when autolysis occurred and reached 125 units /ml at 96 hours.

Specific amylase activity in case of maximal mycelial dry weight was 0.24 in the wild strain and 0.48, 0.83 and 0.88 in mutants Nr. 212, Nr. 514 and Nr. 518 respectively. While *p*H in the wild strain was common to increase to 8.0, in the first phase of mutants it was increased to the similar value, but in the second phase decreased from 8.0 to 5.2 and increased to 8.0 again.

3. Growth in hydrolysed starch substrate

Submerged cultures of mutant Nr. 518 and the wild strain in hydrolysed starch substrate are shown in Fig. 2. Mycelial weight was increased to 174mg MDW/10 ml at 36 hours in mutant Nr. 518 and 189mg MDW/10ml at 48 hours in the wild strain.

Amylase activity was increased to 125 units/ml at 48 hours in a mutant Nr. 518

Table 1. Amylase production in the stationary liquid culture

Mutants	Incubation time (days)						
	2	3	4	5	6	7	8
Wild	12	113	160	220	228	280	250 (E)
	45	153	178	143	135	130	124 (MDW)
	0.3	0.8	0.9	1.5	1.7	2.2	2.0 (E/MDW)
206	11	113	150	210	213	241	240
	53	148	169	153	135	138	118
	0.2	0.8	0.9	1.4	1.6	2.0	2.0
207	7	92	140	200	225	241	235
	38	126	175	153	135	138	118
	0.2	0.7	0.8	1.3	1.7	1.8	2.0
208	5	20	38	160	183	214	205
	20	—	90	130	148	127	128
	0.3	—	0.4	1.2	1.2	1.7	1.6
210	8	97	155	175	219	214	230
	27	128	173	152	126	133	128
	0.3	0.8	0.9	1.2	1.7	1.7	1.8
212	2	43	73	180	239	268	275
	16	52	139	127	125	110	108
	0.1	0.6	0.8	1.4	1.9	2.4	2.6
511	10	102	140	195	240	214	220
	46	142	161	159	139	127	123
	0.2	0.7	0.9	1.4	1.5	1.7	1.8
513	7	99	150	225	210	241	260
	26	137	158	130	130	117	120
	0.3	0.7	0.9	1.7	1.6	2.0	2.1
514	6	105	163	243	250	295	285
	32	161	168	150	140	127	115
	0.2	0.7	1.0	1.6	1.8	2.3	2.5
518	7	75	140	190	225	215	270
	33	135	180	142	136	121	123
	0.2	0.6	0.8	1.3	1.7	1.8	2.2
519	6	113	145	185	225	235	235
	30	155	145	141	136	133	135
	0.2	0.8	1.0	1.3	1.7	2.0	2.0
520	7	102	150	239	233	258	260
	32	126	171	155	143	120	140
	0.2	0.8	0.9	1.5	1.6	2.1	1.9

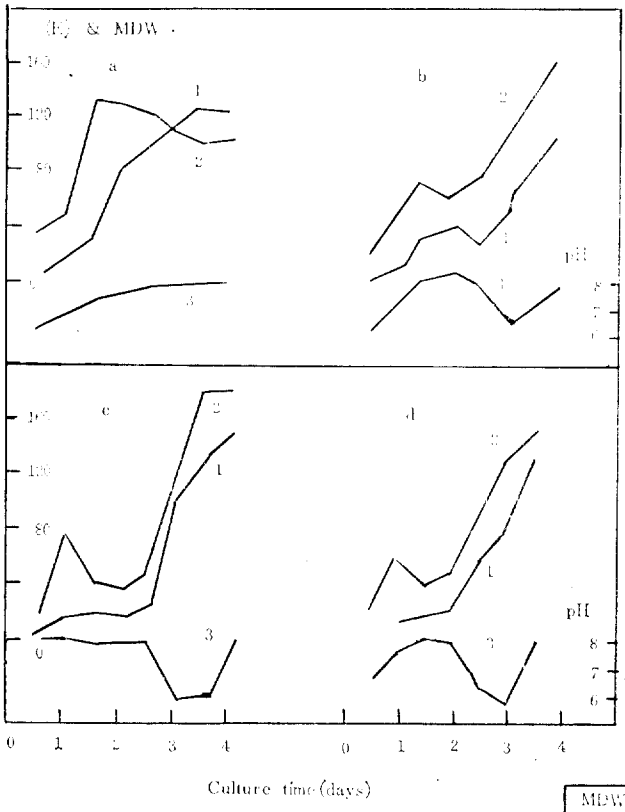

Fig. 1. Growth and amylase productionin submerged cultures in nonhydrolysed starch substrate. 1: (E), 2: MDW, 3: *p*H. a: the wild strain, b: mutant Nr. 212, c: mutant Nr. 414, d: mutant Nr. 518.

and 120 units/ml at 88 hours in the wild strain.

It is characteristic that two-phase of growth did not appeared in hydrolysed starch substrate.

Instead, cultivation time was shortened about 48 hours and amylase production time was reduced *ca.* 40 hours, maintaining similar amylase activity with the wild strain.

Mycelial weight in the wild strain was considerably increased in the hydrolysed starch substrate. Growth phase did not show any difference between hydrolysed starch substrate and nonhydrolysed one in the wild strain.

4. Effect of gibberellin in amylase production

Fig. 3 shows the development of cultures after addition of 25 ppm gibberellin into production medium.

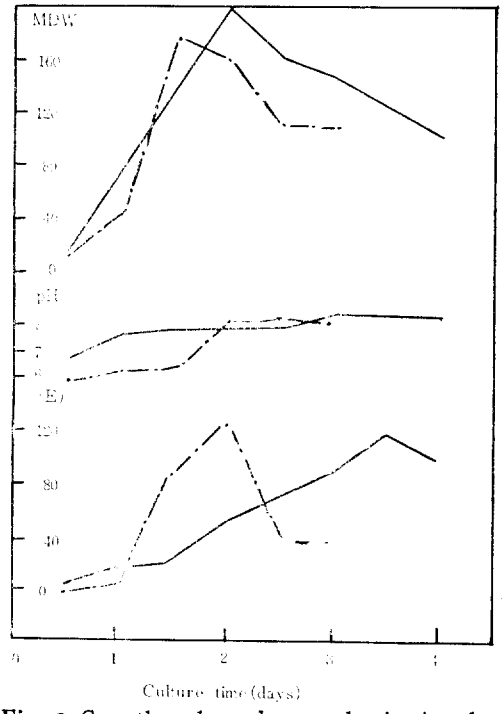

Fig. 2. Growth and amylase production in submerged cultures in hydrolysed starch substrate. Dotted line: mutant Nr. 518, black line: the wild strain.

This concentration was chosen since amylase activity was maximal value at this concentration in surface cultures of liquid medium.

Amylase activity was increased only in stationary liquid culture, and decreased in shaken culture of mutant Nr. 518. Mycelial dry weight was almost less than in non-treatment of gibberellin in either surface culture or shaken culture.

It is observed that the effect of gibberellin is dependent on culture methods and strains.

5. Two-stage continuous culture

As shown in Fig. 4, the batch culture in the first vessel was carried out for 56 hours, thereafter dilution was done at a rate of 75ml/hr.

The speed of agition was 1,000 rpm in the initial batch culture and transferred to 2,000 rpm at start of dilution. Sterile air was supplied at a rate of 100ml/*l* of substrate in the batch culture and altered to 225ml/min/*l* of substrate after 192

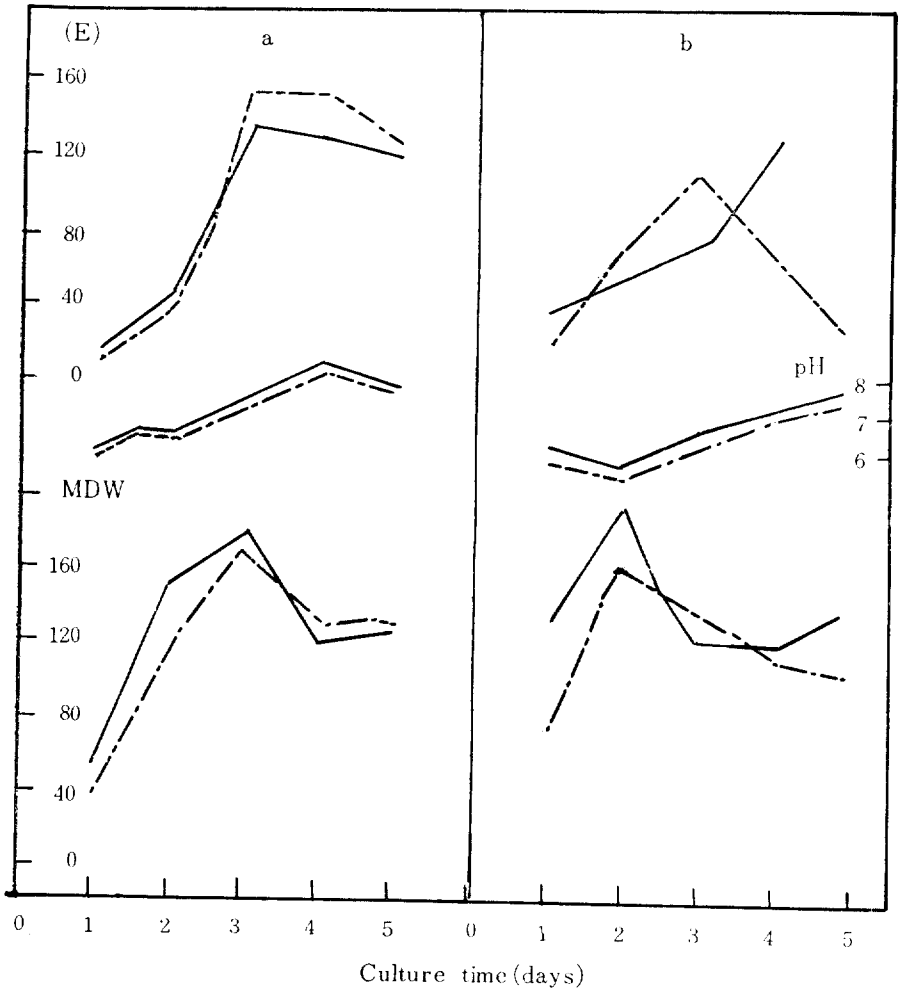

Fig. 3. Effect of gibberellin on amylase production in stationary liquid cultures and shake cultures.

a: stationary liquid cultures, b: shake cultures. 1. dotted line: gibberellin treatment, 2. black line: control.

hours, 200ml/min/*l* of substrate after 336 hours and again 100ml/min/*l* of substrate after 360 hours.

Steady-state amylase production was achieved at 264 hours and maximum amylase titre reached 250 units/ml. Such conditions prevailed for 72 hours.

In the second vessel enzyme titre of effluent reached 250 units/ml at 240 hours and remained at this level for 86 hours. Steady-state enzyme production has proceeded 24 hours earlier than in one-stage culture.

The average volume of culture in each fermenter was 2 litre, which gave a holding time of 26.7 hours per fermenter or 53.3 hours for the whole fermenter.

Enzyme activity was degenerated after 336 hours but still remained at higher level for 504 hours and apparent mycelial weight was not degenerated.

6. Continuous elevation culture

The results are shown in Fig. 5. Elevation culture was started after 108 hours. High enzyme activity as in the batch culture did not reach until 209 hours of the fermentation was developed.

Enzyme activity and mycelial weight were slowly increased to 150 units/ml at 344 hours and 234 mg MDW/10ml effluent at 364 hours respectively.

Enzyme degeneration was not observed unlike the result in two-stage continuous culture. Enzyme activity and mycelial weight showed a strong fluctuation. Holding time was 24 hours in the fermenter.

7. Enzyme productivity in various culture methods

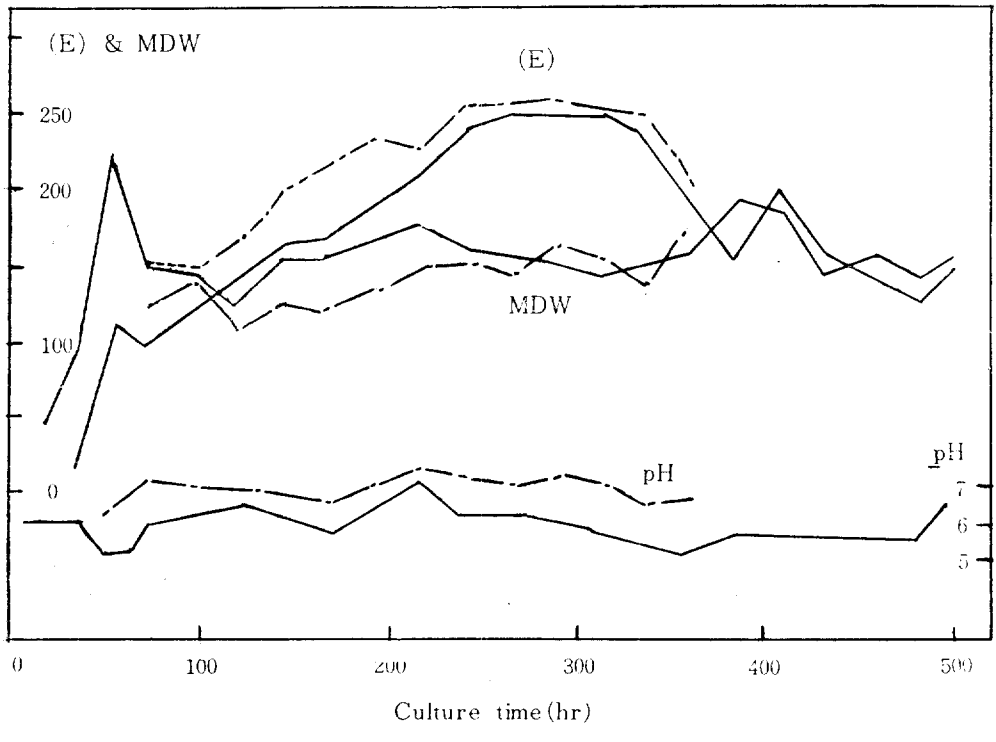

Fig. 4. Two-stage continuous culture in mutant Nr. 518
1. Black line: one-stage continuous culture, 2. Dotted line: two-stage continuous culture

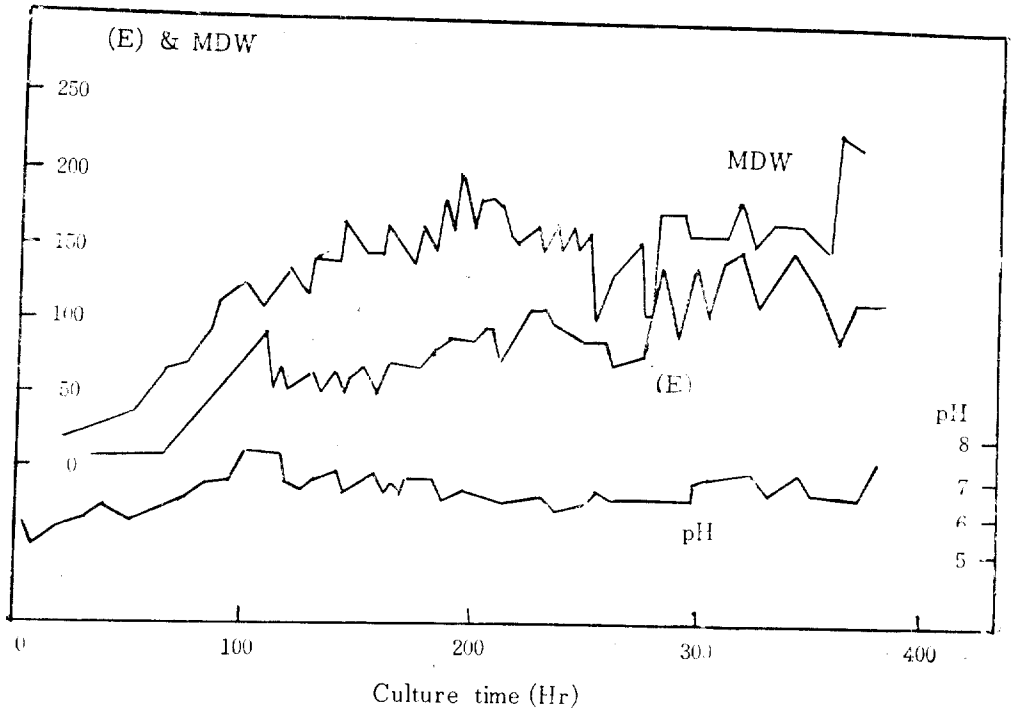

Fig. 5.

Enzyme productivity, P_o can be expressed as the multiplication of volume of output, V and enzyme activity E_n within a unit time, T.

Dimension is units/*l*/hr. That is $P_o = V \times E_n / T$.

Thus the total productivity, P_n is

$$P_n = V \times E_n / T$$

The results are shown in Table 2. The productivity was about 4 times higher in continuous cultures than in batch cultures. In two-stage continuous culture productivity was increased approximately 15% higher than one-stage continuous culture. and was the highest of various methods.

DISCUSSION

A mutant with 35% higher amylase activity was found in *Aspergillus oryzae* after irradiation with 30 krad gamma rays (Gams, 1969). In these experiments

Tablel 2. Comparison with enzyme productivity in various culture methods.

	Continuous		Elevation
	1-stage	2-stage	
Productivity (unit/1/hr)	13.7 (3.9)	15.8 —	8.5 (2.8)
MDW (mg/10ml)	123—191 (219)	122—157 —	107—234 (126)
Culture time (hr)	432 (56)	288 —	272 (108)
Enzyme activity (unit/ml)	110—250 (110)	150—256 —	52—150 (100)
Dilution (ml/hr)	75	75	83.3

( ) : values in a batch culture.

only mutant Nr. 514 showd a little increase. Other 10 mutants were decreased in the amylase activity and mycelial dry weight in suface cultures of liquid media (Table 1).

Enhanced mutants did not reappeared in submerged cultures (Fig. 1, Fig. 2), since it is due to not only chromosomes breakage or abberations but also the difference in the allotment of genetic materials resulted in a parasexual cycle (Hochenhull, 1967; Roper, 1971). Nevertheless, morphological stability was also observed in solid cultures of mutant Nr. 518.

Thus it is considered that irradiation could not be expected to obtain mutants with considerably higher amylase activity.

It is reported that alpha amylase production in *Aspergillus oryzae* var. *microsporus* was increased up to 92% and beta amylase 50% as adding 150 ppm gibberellin to surface cultures of liquid medium (Chung, 1968). Amylase activity of *Aspergillus oryzae* (Alhburg) Cohn used in these experiments was increased 14% at the concentration of 25 ppm gibberellin as optimal concentration in stationary liquid culture (Fig. 3a).

Such an observation was resulted reversely in shaken cultures with a mutant (Fig. 4b). This indicates that gibberellin does not always stimulate both amylase activity and mycelial growth in microorganisms because the effect of gibberellin on microorganisms seems to be probably dependent on strains and culture methods.

It seems to be due to the excretion of necessary substances for amylase synthesis. On agitation of mycelial suspensions of *Mucor japonicus* and *Rhizopus japonicus*, RNA-related nucleotides, mostly mononucleotides, were leaked by mechanical agitation, but not caused by the fracture of mycelia (Tanaka, 1975, 1976).

Treatment of such chemicals as gibberellic acid and indole acetic acid was antagonistic in respiration of each stage of *Chlorella* cells (Chai, 1974; Chung, 1977).

It is of interest that in isolated mutants are shown biphase in growth curves in strongly agitation aeration batch cultures (Fig. 1). Such a phenomenon is reported that in small inocula of unwashed mycelium in shake cultures there is a tendency to autolyse and restart fresh growth before stationary peak is reached which is most pronounced with seeding material taken from an old culture (Meyrath, 1963).

The first phase of growth could be eliminated by replacing with enzyme hydrolysed starch substrate, so that amylase production time was shortened about 40 hours and specific enzyme activity was increased 3 times higher than in submerged cultures before autolysis occurs (Fig. 2). It can be said that mutants have advantages of rapid initial development (large inocula) and those of high specific amylase activity (small inocula) in the performance over mutants (Meyrath, 1971).

The question still remains why amylase activity is reduced down to less than a half value when mutants and the wild strain are cultured in agitation-aeration conditions (Fig. 1, Fig. 2). Amylase production in fungi greatly depends on methods of cultivation (Meyrath, 1971) and probably even on the shape of vessels in solid cultures (Hegyi, 1970). They have found the similar results which amylase activity is remarkably decreased in shake cultures and submerged cultures than in surface cultures.

Reduction of amylase production was not resulted from damage of mycelial growth, since mycelial fracture did not observed in strong agitation using a paddle with a sharp blade. It is rather caused to leak RNA-related mononucleotides by agitation (Tanaka, 1975) and release carbon dioxide in continuous cultures (Gandhi, 1975).

Nevertheless the amylase activity was far more enhanced by the introduction of two-stage continuous culture and continuous elevation culture, although its activity was not exceeded one of surface cultures.

In particular two-stage continuous culture was most effective for amylase production of high level amylase activity, that is, 256 units/ml (one unit corresponds to *ca.* 7.5 Wohlgemuth unit).

Culture degeneration after 350 hours in two-stage continuous culture was observed, because of the fallout of accretion of mycelia inside the fermenter (Means, 1962; Braun, 1972).

In conclusion it should be investigated further more about environmental factors such as agitation, retention of carbon dioxide, fermenter design, culture method and physiological factors in order to operate for a long term industrially.

摘 要

1. 고선량의 '감마선'을 조사하였을때 액체정치배양에서 균사량과 '아미라제' 활성도는 감소가 되고 비 '아미라제' 활성도는 증가되었다.
2. 변이주들은 진탕과 통기배양에서 2개의 생장곡선을 보여주었으며, 생장곡선의 첫번째 곡선은 '아미라제'의 가수분해에 의한 전분기질을 대치함으로써 제거될 수 있었으며, 반면에 효소생성시간은 약 40시간 단축되었고 비 '아미라제' 활성도는 자가분해가 일어나기전에 약 3배가 증가되었다.
3. '아미라제' 생성에 대한 '지베렐린'의 효과는 변이주의 액체정치배양에서만이 양성적인 촉진을 나타냈고 진탕배양에서는 음성적인 효과를 나타냈다.
4. 여러가지 연속배양방법의 실험을 통하여 액체정치배양에서 얻을 수 있었던 '아미라제' 활성도의 수치까지 접근되었을 뿐만 아니라, batch 배양에서 보다 높은 효소 생산성을 얻을 수 있었다.
5. 배양퇴화현상은 2단 연속배양에서 관찰되었으나 연속유가배양에서는 관찰되지 않았다.

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