EFFECT OF ENVIRONMENTAL FACTORS ON THE PRODUCTION OF FUNGAL TANNASE: PART—I

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Tannase activity and growth of different aspergillus strains have been studied under various cultural conditions, eg, composition and pH of the medium, tannic acid concentration, temperature and period of incubation and growth under static or stirred conditions. Temperature influences both tannase activity and mould growth in a similar way. Composition and pH of the medium appear to exert more influence on tannase activity compared to growth. Mould growth is inhibited or restricted by increased tannic acid concentration but tannase production by certain moulds is slightly encouraged. Both tannase activity and mould growth reach to their maximum level more rapidly during stirred culture compared to that of static growth. *A. oryzae*, *A. flavus* and *A. japonicus* are found to produce more tannase compared to other aspergillus strains examined.

Vegetable tan liquors are often contaminated with various moulds, particularly, belonging to the aspergillus and penicillium groups. Tannase produced by such moulds is known to hydrolyse tannic acid to glucose and gallic acid. Hydrolysable tannins are readily attacked by such fungal tannase. Many aspergillus species have been reported[1] to be associated with tan liquors or vegetable tanned leathers but how many of them are capable of producing tannase and to what extent are not clearly known. During primary screening of various strains, Hideaki Yamada *et al*[2] estimated tannase activity of different aspergillus and penicillium strains with the filtrate of culture broth. But during secondary screening of some of the important tannase producing moulds, eg. *A. niger*, *A. flavus*, and *A. oryzae* the tannase activity was estimated with the mycelial extract. They also observed that more enzyme was present in the mycelium than in the broth. In their study, culture was carried out at 30°C with continuous shaking. A variation in cultural conditions was found to influence mould growth as well as tannase production by different investigators.[3-5] Moreover, gallic acid can be conveniently prepared by hydrolysing tannins with suitable fungal strains under optimum conditions of tannase production. The effect of various cultural conditions on the growth and tannase activity of different aspergillus strains has therefore been studied and are reported in this paper.

Materials and methods

Organisms

Various Aspergillus strains, eg. *A. carneus*, *A. ustus*, *A. flavipes*, and *A. japonicus* were obtained from Indian Jute Mills Association, Calcutta; *A. oryzae* from the Department of Botany, University of Madras and *A. niger*, *A. flavus*, *A. nidulans*, *A. parasiticus* and *A. terreus* were isolated in this laboratory.

Medium used

Czapek's liquid medium was used as the basal medium for culturing the fungi. During the assay on tannase, tannic acid was added to the medium in the proportion of 2%, except in certain cases where it has been indicated in the text. Neutral Raulin's medium, malt extract liquid medium and Sabouraud's liquid medium were prepared according to the standard procedures as reported elsewhere.[3] 100 ml. Erlenmeyer flasks containing 25 ml. medium were sterilised at 15 lbs. pressure for 15 minutes. A uniform spore suspension of ten day old culture of the organism was taken and 0.5 ml. of spore suspension was inoculated into the culture medium. The culture flasks were incubated at 30 ± 1°C for about a week. The thick felt, covering the surface in each flask, was separated and used for the determination of mould growth as well as tannase activity.

Estimation of mould growth

The mycelial felt was taken on a filter paper placed over a Buchner funnel. The excess medium was removed by washing the mat (with distilled water) which was then dried to constant weight at 80°C, mould growth was determined from the mat weight and the results obtained (average of two mat weights) are expressed in mg.

Estimation of tannase activity

Tannase activity of *A. oryzae*, *A. flavus*, *A. japonicus*, *and A. niger*, (incubated statically for periods resulting in maximum tannase production) was estimated with both the mycelial extract and the medium. In all cases, tannase activity was found to be significantly higher in mycelial extract.

Mycelial extract was therefore examined for tannase activity.

The mycelial felt was triturated in a mortar with acid washed sand for 5 minutes and then extracted with 10 ml. of double distilled water. The extract was centrifuged at 5000 r.p.m. and the centrifugate was analysed for tannase activity.

Tannase activity was determined by estimating the gallic acid produced during hydrolysis of methyl gallate. The method followed by Yamada *et al*[2] was attempted but due to its rapidity, the method was found to be inconvenient for comparative study.

Dhar[4] standardised the method of estimating gallic acid in presence of methyl gallate by measuring the absorbance at 250 mμ. and 280 mμ. This method was convenient to operate and was followed in the present investigation.

1 ml of the mycelial extract was added to 10 ml. of the substrate solution (10 mg./10 ml. of methyl gallate in 0.5 N acetate buffer of pH 6.0) and the mixture was incubated at 45°C for 24 hrs. Then it was cooled, diluted 100 times with the same buffer and the extinction was determined at 250 mμ and 280 mμ. Control experiments were carried out using boiled enzyme extract. Gallic acid, produced during hydrolysis, was calculated according to the following equation.

$$X_1 = C_1 \alpha_1 + C_B B_1$$
$$X_2 = C_2 \alpha_2 + C_B B_2$$

Where α_1 and B_1 are specific extinction co-effecients of gallic acid and methyl gallate at 250 mμ respectively and α_2 and B_2 are specific extinction co-effecients of gallic acid and methyl gallate at 280 mμ respectively.

1 mg. of gallic acid liberated from 1 ml. of digestion mixture was considered as one unit of enzyme activity.

Results

Effect of media on growth and tannase activity

Spore suspensions of *A. flavus*, *A. niger*, and *A. japonicus* were cultured in four different media : (1) Czapek's medium (2) netural Raulin's medium (3) malt extract medium and (4) Sabouraud's medium. To each of the medium 2% tannic acid was added. The cultured flasks were incubated at 30°C for 7 days. Growth and tannase activity were estimated according to the methods described earlier. The results obtained are given in fig. 1.

Fig. 1 indicates that growth and tannase activity vary considerably depending on the medium used. *A. flavus* and *A. niger* are found to grow better in Sabouraud's medium but in both the cases, tannase activity appears to be more when they are grown in Czapek's medium. In case of *A. japonicus*, however, both the growth and tannase activity are maximum when Czapek's medium is used. It may be further noted that tannase activity is significantly low in case of *A. niger* though moderate mould

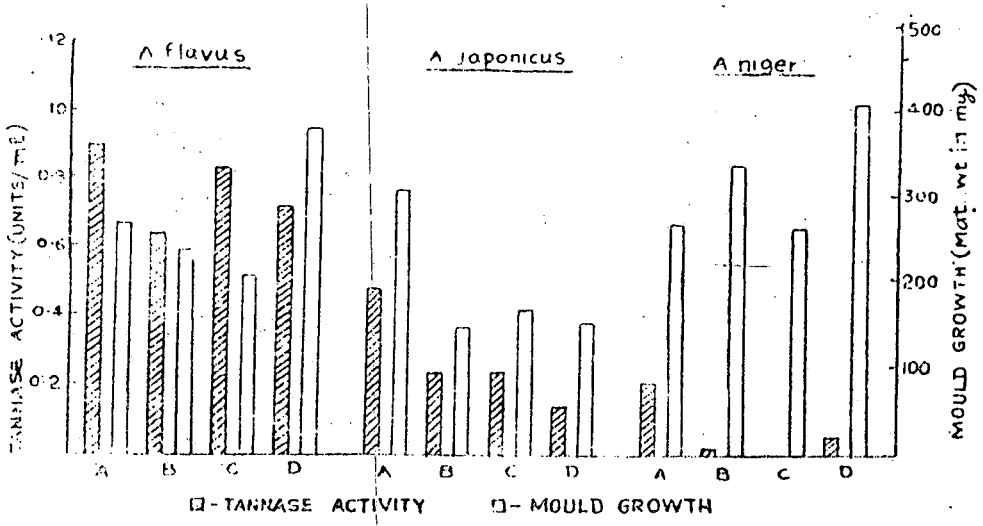

Fig. 1. Effect of media on growth and tannase activity of some Aspergillus species.

A – Czapek's medium
B – Neutral Raulin's medium
C – Malt extract medium
D – Sabouraud's medium

growth takes place in all the media used. The results obtained thus indicate that irrespective of mould growth, tannase production may depend on the composition of the medium and for the Aspergillus species, tannase activity appears to be maximum when they are grown in Czapek's medium. In the following experiments, Czapek's medium has, therefore, been used for the growth of various Aspergillus species.

Effect of tannic acid concentration on growth and tannase activity

Eight Aspergillus species namely *A. flavus*, *A. japonicus*, *A. nidulans*, *A. terreus*, *A. niger*, *A. parasiticus*, *A. oryzae* and *A. flavipes* were cultured in Czapek's medium containing 0.5%, 1%, 1.5% and 2% tannic acid (w/v) and incubated at 30°C for a period of 7 days. Growth of the organism and tannase production by them were estimated and the data obtained are tabulated in Table 1.

A. oryzae appears to be the most sensitive organism towards increasing concentration of tannic acid. Both the growth and tannase activity slightly decrease when tannic acid in the medium increases from 0.5 to 1% but the growth of the organism is practically inhibited at 1.5% tannic acid concentration. Growth of *A. japonicus* and *A. terreus* slightly increases up to 1% tannic acid concentration and then remains unaffected whereas tannase activity by these organisms increases up to 2%. Growth of *A. nidulans* is found to decline but tannase activity increases slightly with the increase in tannic acid concentration. Growth of *A. parasiticus* is affected at 2% tannic acid concentration, but tannase activity remains unaffected. Tannic acid concentration has got no effect either on the growth or tannase activity of *A. niger*. Growth of *A. flavipes*, *A. ustus* and *A. carneus* is appreciably reduced when tannic acid concentration is increased above 0.5%.

Effect of temperature on growth and tannase activity

Spore suspensions of *A. oryzae*, *A. flavus*, *A. niger* and *A. japonicus* were cultured on Czapek's medium containing 0.5% tannic acid in case of *A. oryzae* and 2% in other cases. Culture flasks were incubated at 25°C, 30°C, 37°C and 45°C for a period of 7 days after which the growth and tannase activity of the mycelial extract were determined. Results obtained are presented in Table 2.

It may be seen from Table 2 that most of the mould species examined are influenced in the same way with respect to the growth and tannase activity when they are incubated at different temperatures. 30°C is found to be optimum both for growth and tannase activity with the probable exception of *A. niger* in which case, the growth is found to be unaffected within the temperature range of 25 - 37°C. Growth of *A. oryzae*, *A. japonicus* and *A. niger* was found to be inhibited at 45°C. *A. flavus* could grow slightly at 45°C without sporulation but was incapable of producing tannase at this temperature.

The influence of pH of the medium on growth and tannase activity

Spore suspensions of *A. oryzae*, *A. flavus*, *A. japonicus* and *A. niger* were cultured in Czapeks medium containing tannic acid (2% for *A. flavus*, *A. japonicus*, *A. niger* and 0.5% for *A. oryzae*.) The pH of the media was adjusted to different levels within the range of 2.0 to 6.0. Culture flasks were incubated for seven days at 30°C. Growth and tannase activity were determined as before and the results obtained are presented in Table 3.

TABLE 1

Effect of tannic acid concentration on growth (mat wt. in mg.) and tannase activity (units/ml.) of some Aspergillus species

Mould species	Tannic acid concentration (g./100 ml.) 0.5 G	0.5 TA	1.0 G	1.0 TA	1.5 G	1.5 TA	2.0 G	2.0 TA
A. flavus	295	0.0821	310	0.0820	323	0.0855	326	0.0805
A. flavipes	117	0.0154	27	*	25	*	8	*
A. japonicus	151	0.0302	230	0.0353	210	0.0336	205	0.0438
A. niger	305	0.0133	313	0.0158	311	0.0115	311	0.0128
A. nidulans	295	0.0146	288	0.0187	236	0.0219	219	0.0295
A. oryzae	249	0.0896	229	0.0825	30	*	8	*
A. parasiticus	301	0.0081	305	0.0086	317	0.0092	276	0.0093
A. terreus	222	0.0026	276	0.0071	280	0.0131	278	0.0221

G – growth; TA – tannase activity.
* could not be estimated due to the insignificant growth.

TABLE 2

Effect of temperature on growth (mat wt. in mg.) and tannase activity (units/ml.) of certain Aspergillus species

Mould species		Temperature 25°C	30°C	37°C	45°C
A. flavus	G	261	277	233	36
	TA	0.0785	0.0922	0.0645	**
A. japonicus	G	230	291	112	6
	TA	0.0428	0.0451	0.0362	**
A. niger	G	267	303	303	5
	TA	0.0113	0.0150	0.0078	**
A. oryzae	G	249	301	19	3
	TA	0.1005	0.1104	0.098	**

G—growth; TA—tannase activity
**could not be estimated due to the insignificant growth.

TABLE 3

Effect of pH of the medium on growth (mat wt. in mg.) and tannase activity (units/ml.) of some Aspergillus species

Mould species		*pH*						
		2.5	*3.0*	*3.5*	*4.0*	*5.0*	*5.5*	*6.0*
A. flavus	G	244	320	**	319	310	310	305
	TA	0.0629	0.0397	**	0.0668	0.0633	0.0512	0.0430
A. japonicus	G	216	227	233	239	229	**	236
	TA	0.0309	0.0414	0.0510	0.0295	0.0245	**	0.0117
A. niger	G	**	271	286	295	282	292	289
	TA	**	0.0118	0.0130	0.0096	0.0095	0.0085	0.0064
A. oryzae	G	**	159	236	238	233	**	211
	TA	**	0.0392	0.0773	0.0804	0.0983	**	0.0635

G—growth; TA—tannase activity.

**pH not considered in respective cases.

In all the occasions, growth is found to be very little affected within the pH range 3/3.5 to 5.5/6. But tannase activity is found to be optimum for *A. flavus*, *A. japonicus*, *A. niger* and *A. oryzae* at pH 3.0, 3.5, 3.5 and 5.0 respectively. Of course, the extent of variation within pH value 3.0 to 5.0 in case of *A. niger* is not very significant.

Influence of static and stirred conditions of culture on growth and tannase activity.

Spore suspensions of *A. oryzae*, *A. flavus* and *A. japonicus* were cultured in Czapek's medium, pH being adjusted to 5.0, 3.0 and 3.5 respectively. One batch of cultured flasks was kept in static condition and other batch was put to a continuous shaker and incubated for a period of seven days at room temperature. Growth and tannase activity were determined at regular intervals and the results obtained are presented in fig. 2.

Fig. 2 indicates that both the growth and tannase activity of *A. oryzae* reach to their maximum level after 7 days when grown in static condition and these values are higher than the corresponding maximum values obtained with continuous shaking.

In case of *A. flavus*, however, mould growth appears to be higher but tannase activity lower with continuous shaking. On the other hand, both the growth and tannase activity of *A. japonicus* appear to be higher with continuous shaking. In static culture, tannase activity of *A. japonicus* and *A. oryzae* appears to increase up to 7 days after which it was found to decrease.

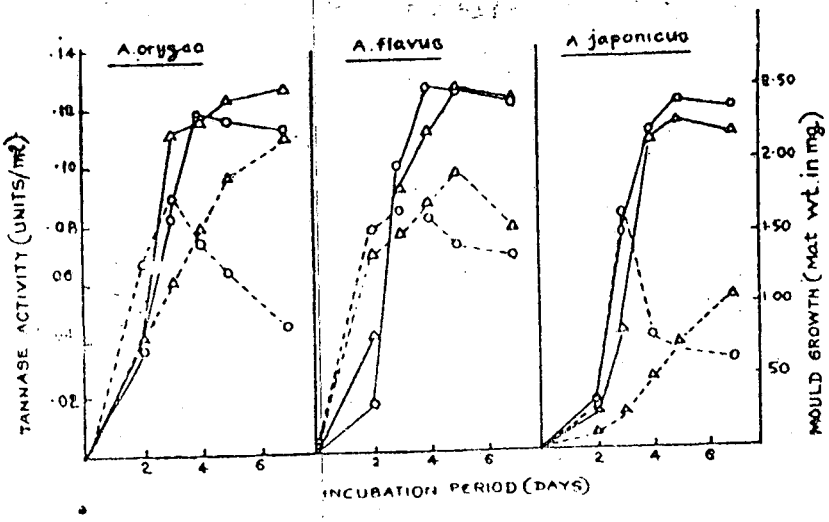

Fig. 2 : The influence of static and stirred conditions on growth and tannase activity of some Aspergillus species.

△——△ Tannase activity in static culture
○——○ Tannase activity in stirred culture
△- - - -△ Mould growth in static culture
○- - - -○ Mould growth in stirred culture

Effect of period of incubation on the tannase activity of different Aspergillus species

Seven Aspergillus strains were cultured in Czapek's medium containing different percentages of tannic acid. 2% tannic acid was incorporated into the medium for *A. nidulans*, *A. terreus*, *A. niger* and *A. parasiticus*, 0.5% tannic acid for *A. ustus* *A. carneus*, and *A. flavipes*. Culture flasks were incubated at 30°C for a period of 13 days and tannase activity was estimated at regular intervals. Results obtained are tabulated in Table 4.

It is evident from Table 4 that tannase activity of different Aspergillus strains increases with the period of incubation and it appears to be maximum after 5 days for *A. nidulans* and 7 days for *A. niger*, *A. terreus* and *A. ustus*. These strains are found to produce more tannase compared to the other three strains, which require 9-11 days for maximum tannase production.

Discussion

The present observations indicate that optimum conditions for the growth of Aspergillus strains may differ from the optimum conditions for tannase production. A similar observation was made by Hirohsi Nishira and Narataro Mugi Bayashi[4] who found no correlation between tannase formation and the mycelial growth of moulds grown on tannin-$(NH_4)_2SO_4$ medium. During primary screening of moulds Hideaki Yamada *et al*[2], however; noted some broad correlation between tannase activity in the culture broth and the growth of the fungus. It is observed from Fig. 1 that mould growth and tannase activity of

TABLE 4

Effect of period of incubation on tannase activity (units/ml.) of some Aspergillus species

Mould species	*Period of incubation (days)*					
	3	*5*	*7*	*9*	*11*	*13*
A. carneus	*	0.0012	0.0014	0.0036	0.0013	*
A. flavipes	*	0.0020	0.0063	0.0110	0.0134	0.0047
A. niger	0.0130	0.0220	0.0240	0.0140	0.0076	*
A. nidulans	0.0247	0.0333	0.0315	0.0250	0.0238	*
A. parasiticus	0.0011	0.0032	0.0054	0.0091	0.0133	0.0123
A. terreus.	*	0.0030	0.0293	0.0276	0.0248	0.0198
A. ustus	*	0.0296	0.0305	0.0238	0.0152	0.0073

*Insignificant/no tannase activity.

the mycelial extract are not directly related in all cases and tannase production by *A. niger* is found to be significantly low in spite of good mycelial growth. The composition of the medium may have greater influence on tannase production. Mould growth as well as tannase production are also influenced by tannic acid concentration in the medium. Growth of *A. oryzae* and *A. flavipes*, is appreciably affected above 0.5% tannic acid. Tannase production by *A. japonicus*, *A. terreus* and *A. nidulans* increases with tannic acid concentration up to 2% though such an increase in mould growth has not been recorded. In general, it has been observed that tannase production slightly increases or remains unaffected but the growth of the mould species examined is either inhibited or affected very little with the increase in tannic acid concentration.

As regards temperature, both the growth and tannase activity of the Aspergillus strains are found to be optimum at 30°C. According to Masao oder *et al*[4] the optimum temperature for tannase production by *A. japonicus* and *A. oryzae* was 30-37°C and 37°C respectively.

"Growth of Aspergillus strains is less affected by pH variation but tannase activity is appreciably affected. In the present investigation, pH 3.0 is found to be optimum for tannase production by *A. flavus*. Hideaki Yamada *et al*[2] however, observed that enzyme activity of purified tannase from *A. flavus* was optimum at pH 5.0. The optimum pH for tannase production by *A. japonicus*; *A. niger* and *A. oryzae* appears to be 3.5, 3.5 and 5.0 respectively. According to Masao oder *et al*[4] both *A. japonicus*, and *A. oryzae* showed optimum tannase activity at pH 5.0. pH 4.0 was reported by Dhar[5] to be the optimum for tannase production by *A. niger*".

It is apparent from Fig. 2 that the rate of mould growth and tannase production

vary considerably during static and stirred conditions of growth. In stirred conditions both the growth and tannase activity reach their maximum level much earlier than in static condition. Hideaki Yamada *et al*[2] observed maximum tannase production by *A. flavus* after 24 hrs. of growth in stirred condition. In the present study, more tannase is found to be produced by *A. flavus* in static condition compared to stirred condition. In case of *A. flavus* and *A. oryzae* static condition encourages tannase production but in case of *A. japonicus*, stirred condition is found to favour the same.

Table 4 reveals that other Aspergillus strains are capable of producing small quantity of tannase. This appears to be significantly low when compared to *A. oryzae*, *A. flavus* and *A. japonicus*. Tannase production by *A. corneus*, *A flavus*, and *A. parasiticus* is found to be insignificant.

It may, however, be emphasised that growth as well as tannase activity of different species of the same mould may vary considerably as might be evident from the observations made by Hideaki Yamada *et al*[2]. Considering the above observations *A. oryzae*, *A. flavus* and *A. japonicus* are found to produce more tannase than the other Aspergillus strains studied.

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