PRESLIA 1963
35 : 185—192

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A Comparison of Different Aspergilli With Regard to the Amylolytic Activity of Their Enzymes

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The production of hydrolytic enzymes by microorganisms is considered as an important characteristic in their taxonomy and as a valuable property from the practical standpoint. Some lower fungi are good producers of industrially prepared enzymes, e.g. α-amylases, polygalacturonases, proteases, cellulases and lipases. Several more or less extensive selections and evaluations of different species and of different strains of the same species have been performed in this direction. All authors are in agreement as to the great variability of the amount of produced enzyme not only between different species, but also between different strains (Le Mense et al. 1947, Dingle et Solomons 1952.) The final amount of produced enzyme resp. its activity depends of course on the composition of the medium where the moulds are grown and on other conditions of cultivation. Optimal conditions are usually different for different strains of the same species (Tsuchia et al. 1950, Stárka 1953). The main criterion considered in the selection of appropriate strains is the activity of the studied enzyme expressed e.g. as amount of reducing sugars in enzymatic hydrolysate of starch when reaction equilibrium has been reached. In this study an attempt has been made to analyze in some detail the activity of a starch-splitting enzyme system of 17 strains belonging to 8 different species of Aspergilli. It was supposed that characteristic differences could be expected rather in the dynamics of enzymatic hydrolysis of starch (Stárka 1955) than in the total quantity of reducing sugars formed.

In comparing our strains the following estimations were made:

1. formation of reducing sugars in course of hydrolysis
2. kinetics of viscosity during hydrolysis of starch
3. formation of glucose, maltose and other oligosaccharides (quantitative determination)
4. production of ethanol in fermented enzymatic hydrolysate of starch.

Materials and methods

For the analysis of the amylolytic enzyme complex 17 strains of the following species of Aspergilli were used:

Aspergillus oryzae (2 strains)
Aspergillus flavus
Aspergillus niger (5 strains)
Aspergillus wentii
Aspergillus terreus (3 strains)
Aspergillus violaceo-fuscus
Aspergillus fumigatus (3 strains)
Aspergillus nidulans (2 strains)

All strains are from the collection of our laboratory. Strains were maintained on wort agar slants and reinoculated every 4 weeks. Suspension of spores was prepared by washing one agar tube with 100 ml of water. Cultivation on bran was performed as described earlier (Stárka 1952). After 4 days of cultivation mould bran was dried on filter paper at 20°, ground and stored in well stoppered bottles.

Enzymatic extract was prepared by elution of 5 g of dry mould bran in 100 ml water at 30° under continuous mixing and by filtration after 60 min. 2 ml of enzymatic extract were added to 10 ml of 2% starch in acetate buffer pH 4,5 and incubated at 30°.

Activity of enzymatic extract was measured iodometrically (SOMOGYI 1945) and dextrinogenic activity was estimated by modified Wohlgemuth method (STÁRKA 1953). Decrease of viscosity of starch solution was measured by Ubbelohde viscosimeter. Filtered 5% extract of mould bran was diluted 1 : 10 and thoroughly mixed; extimation of viscosity occurred at 30°. For chromatographic analysis of starch hydrolysis by mould enzymes 5% water extract of mould bran was mixed with 2% starch solution in proportion 1 : 10 and incubated at 30°. Samples were taken at 10, 30, 60 and 120 min., inactivated by boiling for 2 min., cooled and immediately examined by chromatography using Whatman no. 1 paper. Descending technique in the butanol-pyridine-water (3 : 2 : 1,5) solvent was employed. After the solvent descendend to the lower edge of the sheet, the chromatogram was dried and the process was repeated. The spots were detected by spraying with 0,05% diphenylamine in 10% trichloracetic acid and drying at 110°. The location of spots was determined by visual inspection in ultraviolet light. For quantitative determination two parallel samples were applied to the paper and run in the solvent, after which the one was detected and the other used for elution and subsequent colorimetric determination of sugars by anthrone method described previously (STÁRKA 1955).

For fermentation tests, dried and powdered potatoes containing approximatively 66% starch were used. A quantity corresponding to 10 g of starch was dissolved in 200 ml of hot water and after cooling to 55° supplemented with mould bran (0,5 and 1,0 g of dry weight). Saccharification occurred 60 min. at 55°, then the hydrolysate was cooled to 27° and adjusted to pH 5,2 with M/5 H_2SO_4. Each flask was inoculated with 2,5 g of fresh distillers' yeast, fitted up with rubber stopper and bubbling tube and incubated at 27° for three days. Alcohol content was then determined pycnometrically after distillation. The blank was arranged similarly but without adding mould bran. Blank value was subtracted from final result of fermentation test, expressed in volume % of alcohol.

Results

The course of enzymatic hydrolysis of starch is summarized in Table 1, where the formation of reducing sugars and dextrinogenic activity of studied strains is given. Highest values of reducing sugars were obtained in samples,

Table 1

The determination of reducing sugars in enzymatic hydrolysate of starch

Strain	Reducing sugars in mg.*			Achromatic point (min.)
	10 min.	30 min.	60 min.	
A. oryzae F 1	5,12	6,99	7,47	60
A. oryzae S 1	4,63	6,34	6,89	60
A. flavus B 1	5,30	6,32	6,50	120
A. niger F 4	6,12	6,42	6,58	120
A. niger F 6	6,23	6,81	6,89	—**
A. niger F 8	6,25	6,84	6,92	120
A. niger F 9	6,21	6,29	6,39	120
A. niger F 5	6,32	6,86	6,95	120
A. wentii F 3	5,54	6,11	6,34	—
A. violaceofuscus F 16	1,73	3,63	5,85	—
A. terreus F 17	1,47	3,94	6,47	120
A. terreus F 18	2,53	5,22	6,38	60
A. terreus 227	1,62	4,11	5,98	60
A. fumigatus F 12	1,83	3,82	4,72	—
A. fumigatus 231	2,02	4,11	4,52	—
A. fumigatus F 14	2,47	4,42	4,81	—
A. nidulans F 11	1,22	2,69	3,28	—
A. nidulans F 10	0,71	2,48	3,32	—

* Reducing sugars expressed as glucose.

** Achromatic point was not reached even after 180 min. of hydrolysis.

where *Aspergillus niger*, *Aspergillus wentii* and *Aspergillus oryzae* were used. Also *Aspergillus terreus* and *Aspergillus violaceofuscus* hydrolyze starch to greater quantity of reducing sugars, but the process is slower. With strains of *Aspergillus fumigatus* and *Aspergillus nidulans* the values of reducing sugars were small even after 60 mins. of hydrolysis.

The differences in liquefying activity of strains belonging to the same species were negligible. How individual species differed is shown in Fg. 1. Most rapid decrease of viscosity was observed in samples with extracts of *Aspergillus oryzae*, *Aspergillus flavus* and *Aspergillus terreus*. The group *Aspergillus niger* — *Aspergillus wentii* liquefied starch more slowly, other Aspergilli were even less active.

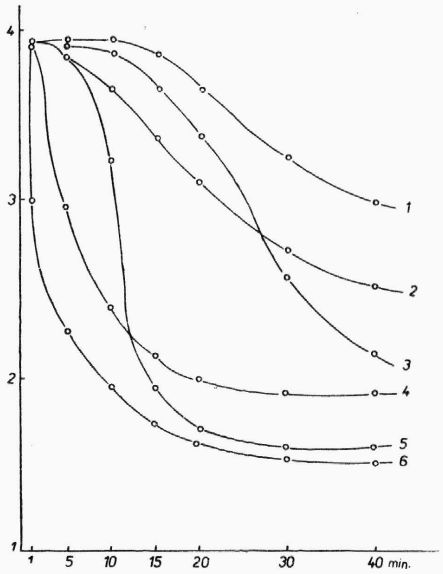


Fig. 1. Liquefying activity of different Aspergilli. 1 = A. fumigatus F 14, 2 = A. nidulans F 10, 3 = A. violaceofuscus F 16, 4 = A. terreus F 18, 5 = A. niger F 6, 6 = A. oryzae F 1. The differences between various strains of the same species were negligible. On the ordinate relative degrees of viscosity.

Accumulation of glucose, maltose and oligosaccharides was followed quantitatively by means of paper chromatography. The most remarkable activity showed strains of *Aspergillus niger*, *Aspergillus wentii*, *Aspergillus oryzae* and *Aspergillus terreus*. In the case of *Aspergillus niger* and *Aspergillus wentii* a large amount of glucose and only traces of maltose and oligosaccharides are formed. Strains of *Aspergillus oryzae* differed in glucose: maltose ratio and in high proportion of oligosaccharides. Six distinctly separated spots of non-identified oligosaccharides other than maltose were observed, *Aspergillus flavus* gave an intense spot of glucose and a relatively high proportion of oligosaccharides but the quantity of maltose was low. Similar results were observed with *Aspergillus terreus*. Other strains (*Aspergillus violaceofuscus*, *Aspergillus nidulans* and *Aspergillus fumigatus*) hydrolyzed starch preponderantly to glucose (fig. 2.).

Quantitative estimation of the time-course of starch hydrolysis by means of paper chromatography showed again that the differences in kinetics between the representatives of one species were unimportant but that the differences between species examined were significant. These observations are shown on fig. 3. An interesting difference exists between *Aspergillus oryzae* and *Aspergillus flavus* in the maltose: glucose ratio, although both species are closely related. On the other hand, the hydrolytic activities of *Aspergillus niger* and *Aspergillus wentii* are in good agreement. Figs. 4, 5, 6 and 7 (see Plate XVI) depict the separation of glucose, maltose and oligosaccharides on chromatograms in the course of starch hydrolysis by preparations of *Aspergillus flavus*, *Aspergillus oryzae*, *Aspergillus niger* and *Aspergillus terreus*, respectively. An interesting comparison can be made when the results of quantitative estim-

ation of the dynamics of starch hydrolysis by enzymatic preparations of individual strains are summarized. Table 2 shows quantitative ratios of individual saccharides from different preparations after 120 min. of hydrolysis.

These values again give evidence for fundamental difference between the

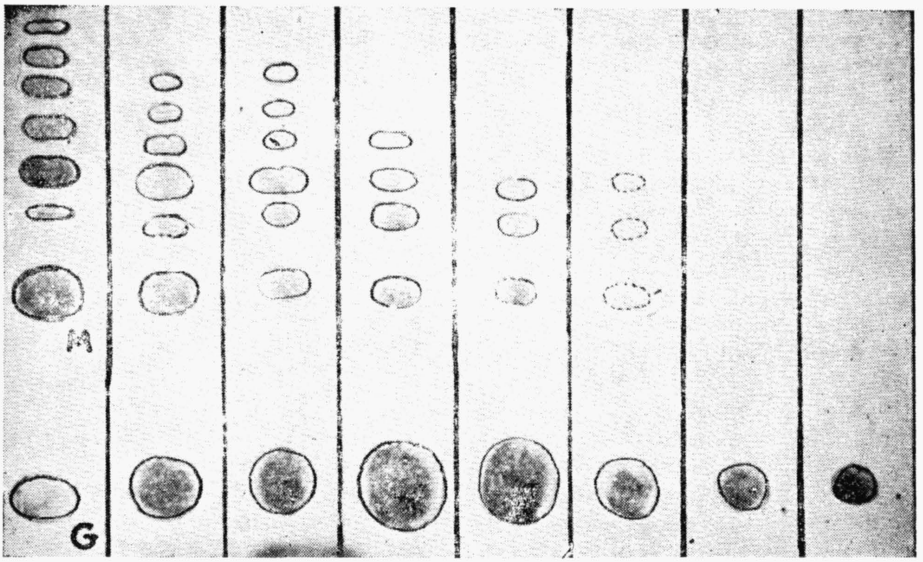


Fig. 2. Chromatographic analysis of sugars in enzymatic hydrolysate of starch formed by different strains of Aspergilli. From the left: A. oryzae F 1, A. flavus B 1, A. terreus F 18, A. niger F 6, A. wentii F 3, A. violaceofuscus F 16, A. fumigatus F 14, A. nidulans F 10. G = glucose, M = = maltose, incubation 120 min.

Table 2

Glucose : maltose : oligosaccharides ratio after 120 min. of hydrolysis of starch by enzymes of Aspergilli

Strain	Glucose*	Maltose*	Other oligo-saccharides*	G : M : O ratio
A. oryzae F 1	52	84	95	5 : 8 : 10
A. oryzae S 1	51	79	96	5 : 8 : 10
A. flavus B 1	78	52	92	8 : 5 : 9
A. niger F 6	182	35	77	9 : 2 : 4
A. niger F 9	186	31	65	9 : 2 : 3
A. wentii F 3	196	58	76	10 : 3 : 4
A. violaceofuscus F 16	132	11	30	13 : 1 : 3
A. terreus F 17	166	53	133	17 : 5 : 13
A. terreus F 18	158	48	93	16 : 5 : 9
A. fumigatus F 12	52	0	0	5 : 0 : 0
A. fumigatus F 14	61	0	0	6 : 0 : 0
A. nidulans F 10	32	0	0	3 : 0 : 0

* Values obtained from eluted spots and expressed as glucose in μg.

group of *Aspergillus oryzae* and *Aspergillus niger*. When the activity of the amylolytic system is considered, *Aspergillus oryzae*, *Aspergillus flavus* and to a certain degree *Aspergillus terreus* are closely related. This group is characterized by the accumulation of a relatively high amount of oligosaccharides, whereas the quantity of glucose is low. The *Aspergillus niger* group (to which A. *wentii* and perhaps *A. violaceofuscus* belong) splits starch typically to glucose and oligosaccharides are formed in a relatively small amount.

Table 3

Yield of ethanol from fermented hydrolysates of starch

Strain	Yield in vol. % of ethanol	
	0,50 g*	1,00 g*
A. oryzae F 1	5,268	6,072
A. flavus B 1	4,756	6,00
A. niger F 6	4,923	6,510
A. wentii F 3	5,815	6,616
A. terreus F 18	1,244	1,963
A. terreus F 17	0,662	1,466
A. fumigatus F 12	2,463	4,213
A. fumigatus F 14	1,988	3,197
A. nidulans F 10	2,221	4,243
A. nidulans F 11	2,162	3,420

* Dry weight of mould bran used for hydrolysis.

The fermentation test gave another interesting comparison of strains studied. In this case the enzymatic hydrolysate of starch was fermented by distillers' yeast to ethanol. The yield of alcohol corresponds to the actual quantity of fermentable sugars (tab. 3). Best results were obtained with a preparation of *A. niger* and *A. wentii*, but *A. oryzae* and *A. flavus* were also satisfactory.

Discussion

Klimovskij and Rodzevič (1950) divided their 20 strains of Aspergilli in two groups according to their starch-splitting activity. The representatives of these groups were respectively *A. niger* and *A. oryzae*. The analysis of several strains of Aspergilli, carried out in some more detail in the present paper, evidenced the inter-species and inter-group differences. The most interesting results were obtained in the experiments where the dynamics of the formation of glucose, maltose and oligosaccharides by amylolytic enzymes of different strains was estimated. It is clear that the individual activity of transglucosidic enzymes of each strain plays an important rôle. This was demonstrated in our precedent paper (Stárka 1955) where *A. niger* and *A. oryzae* were compared. Similar results were obtained by Beran and Burger (1956) vith *A. niger*.

An interesting relation appears to exist between the liquefying capacity of the enzymatic preparations and the fermentability of hydrolysate to alcohol by yeast. *A. niger* liquefies starch solution more slowly than *A. oryzae*, but it yields more alcohol. On the other hand, the yield of alcohol is not always in agreement with the amount of reducing sugars formed. *A. terreus* and *A.*

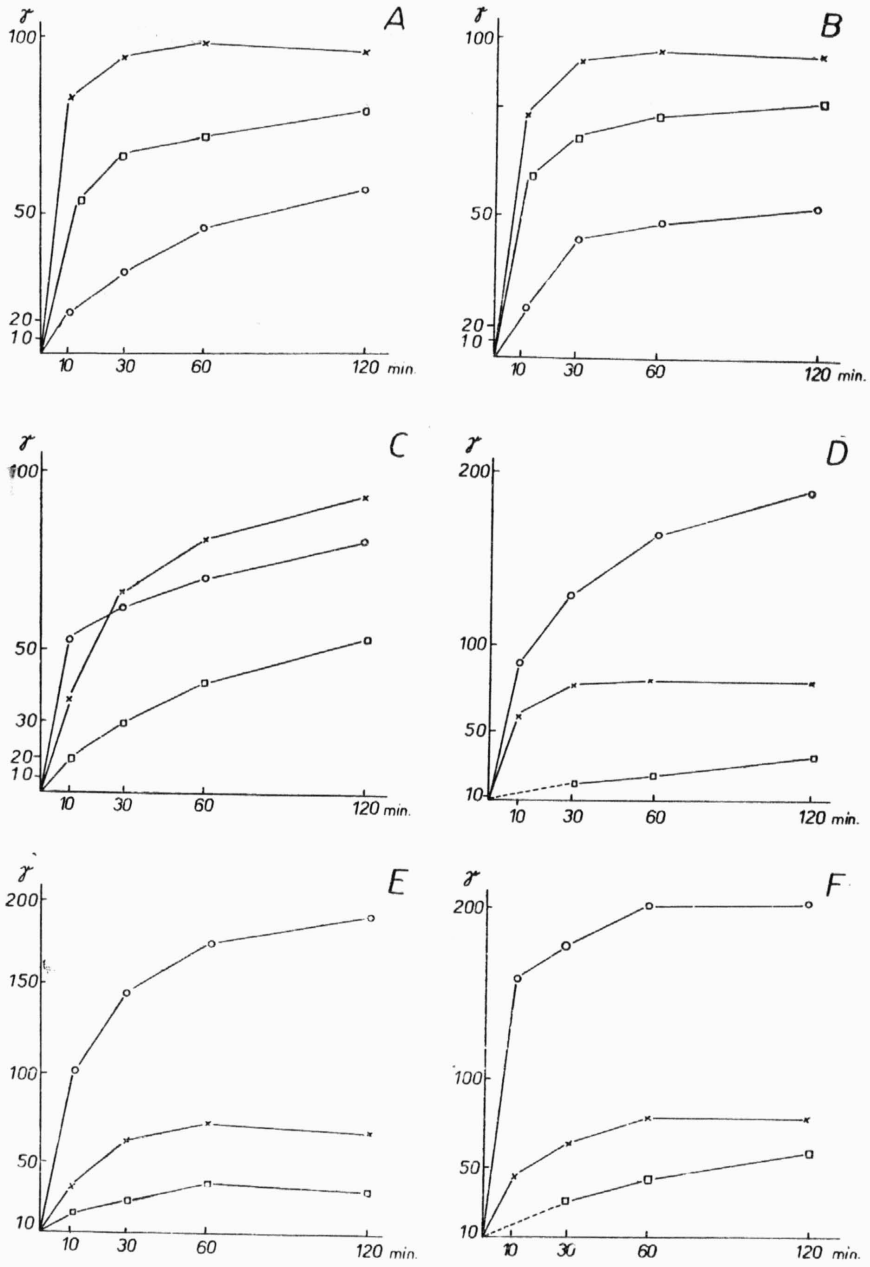
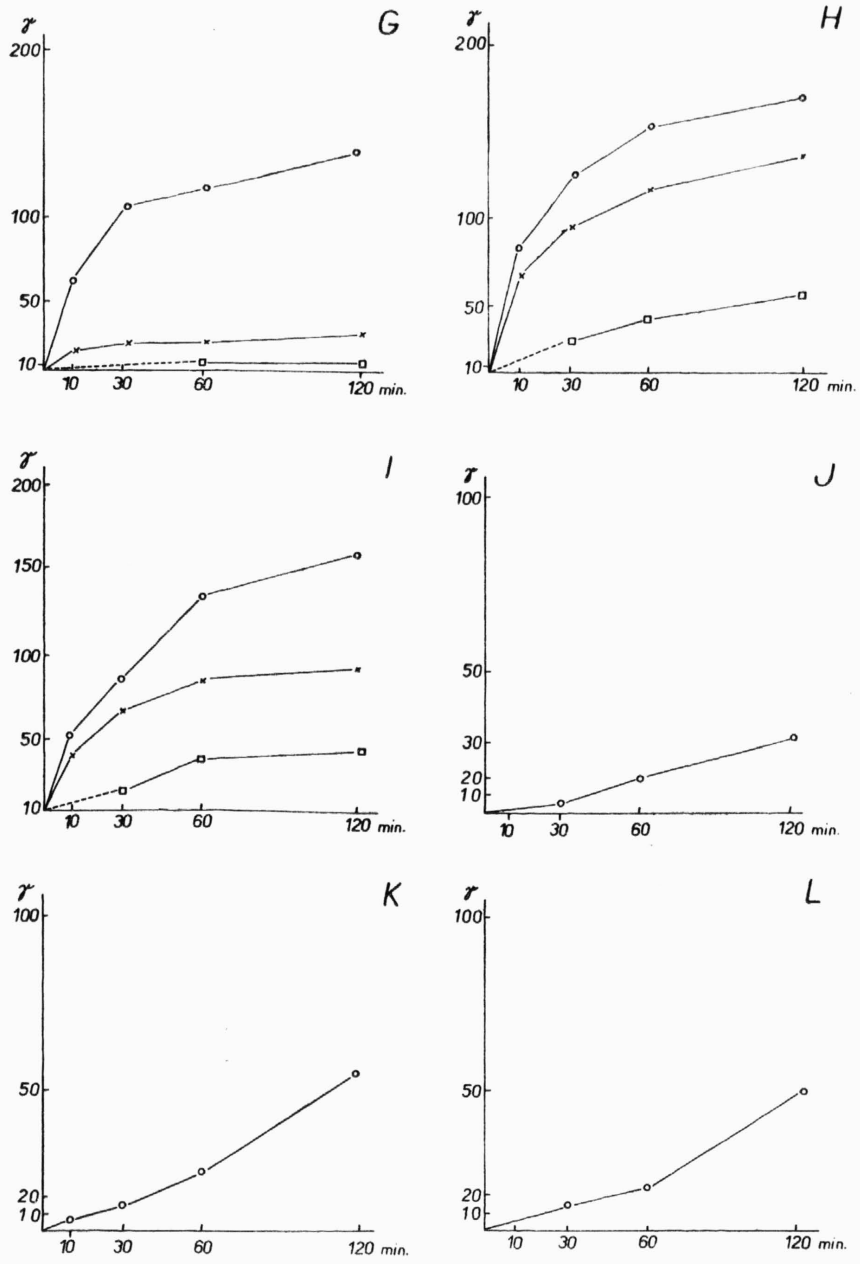


Fig. 3. Dynamics of starch hydrolysis by enzyme preparations from various strains of Aspergilli estimated by quantitative chromatography on paper. From the top left: A. oryzae S 1 (A), A. oryzae F 1 (B), A. flavus B 1 (C), A. niger F 6 (D), A. niger F 9 (E), A. wentii F 3 (F), A. viola-



ceofuscus F 16 (G), A. terreus F 17 (H), A. terreus F 18 (I), A. nidulans F 10 (J), A. fumigatus F 14 (K), A. fumigatus F 12 (L). ○ = glucose, □ = maltose, × = other oligosaccharides (total). Ordinate: γ of sugars expressed as glucose.

niger produce practically the same quantity of reducing sugars, but the yield of ethanol from hydrolysates obtained with *A. terreus* is much inferior. The only reliable indication for strains, which might be useful for saccharification of starch-containing mashes in the industrial production of alcohol, appears to be the fermentation test.

The number of strains compared in this paper does not allow drawing conclusions as to the relation between the position in the system and enzymatic activity of the studied species of Aspergilli. It should be noted that other authors (e. g. TSUCHIYA et al., 1950) found much higher variation of amylolytic activity when working with a large number of strains of the same species. These results are not contradictory to our observations. The variation of enzymatic activity, i. e. of the rate of synthesis of a given enzyme, is a common property of the members of the same species. The quantitative differences in the final enzyme production (i.e. units of enzyme /unit of dry weight) are not species-specific, at least in the case of Aspergilli. But the analysis of the amylolytic enzymes presented in our paper indicate quantitative differences which seem to be species-specific or group-specific. It would be interesting to apply this analysis to a greater number of representatives of the same species and to other enzymes.

Summary

An analysis of amylolytic enzyme complex of 17 strains belonging to 8 species of the genus *Aspergillus* is described. Dynamics of the formation of sugars is greatly influenced by transglucosidic activity of saccharidases of individual species. The quantitative differences seem to be species-specific or group-specific. The methods described appear to be promising tool for systematic studies.

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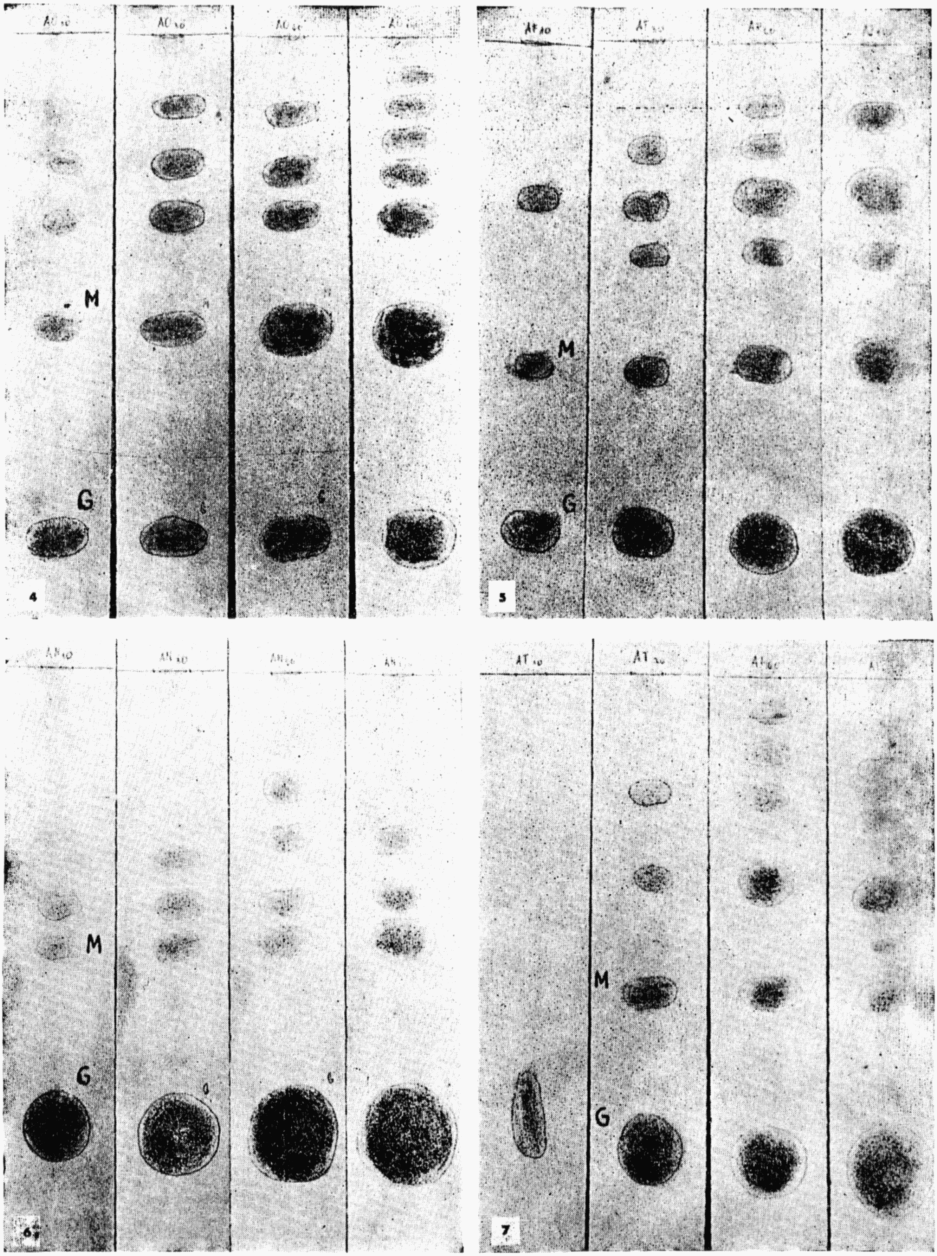
Jiří Stárka a Hana Závadová:

Srovnání amylolytické aktivity enzymů u různých druhů rodu *Aspergillus*

Je popisována analysa amylolytického enzymového komplexu 17 kmenů patřících k osmi druhům rodu *Aspergillus*. Dynamika tvorby redukujících cukrů je významně ovlivněna transglukosidickou aktivitou sacharidáz jednotlivých druhů. Kvantitativní rozdíly jsou patrně druhově nebo skupinově specifické. Popsané metody mohou být užitečné pro systematické studie.

Explanations of the plate:

Plate XVI: Figs. 4, 5, 6 and 7. Distribution of glucose, maltose and unidentified oligosaccharides on chromatograms at different intervals of hydrolysis of starch by preparations from A. oryzae F 1 (4), A. flavus B 1 (5), A. niger F 6 (6) and A. terreus F 17 (7) after 10, 30, 60 and 120 min. G = glucose, M = maltose.



J. Stárka and H. Závadová: A Comparison of Different Aspergilli With Regard to the Amylolytic Activity of Their Enzymes