The Effect of Carbohydrates on Growth and Sporulation of Aspergillus flavus and their carry over for subsequent Spore Germination

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The great differences in the ability of fungal spores to germinate when obtained from various sporulation media indicated the importance of judicious selection of culture media for raising the cultures for any specific work (Darby and Mandels, 1955; Grover, 1964; Allen, 1965; Kumar and Grover, 1967). Cochrane et al. (1963) suggested that the insufficiency of carbon reserves in the fungal spores is accomponied by a requirement of specific respiratory intermediate not available in the spore reserve.Whereas Caltrider, Ramachandran and Gottlieb (1963) and Caltrider and Gottlieb (1966) pointed out that the endogenous substrates are preferentially utilized during the spore germination in case of rust and smut fungi. Obviously, the spore reserves are a critical factor in their future metabolic activity.

Basic physiological and biochemical data concerning the formation of spore reserves are sparse (Sussman and Halvorson, 1966). The importance of sporulation medium upon subsequent behaviour in germination and metabolic activity of spores has been emphasized by Cochrane (1960) and Allen (1965). In an earlier study data concerning the role of amino acids in the substrate media on subsequent spore germination of *Aspergillus flavus* Link ex Fries indicated the essentiality of atleast five amino acids, whose presence in the basal medium was thought to be essential for normal metabolism of spore germination (Grover, 1964). The present account describes the effect of some of the carbon sources on growth sporulation and their carry-over for subsequent spore germination of *A. flavus.*

Materials and Methods

The isolate of *Aspergillus flavus* was the same as used earlier (Grover, 1964). The basal medium consisted of KH_2PO_4 5 g; $MgSO_4 . 7H_2O$ 2.5 g; KNO_3 10 g; $FeCl_3$ 0.01 g; and distilled water to make 1 litre. The carbon sources were added to the basal medium in amounts calculated to give equivalent concentration of carbon. The carbon compounds were sterilized separately and then added to the already sterilized basal medium. The pH of all the media was adjusted to 6.0. The inoculum used

(1 ml containing 1×10^3 spores from 8—10-day-old cultures) was made from cultures growing on the basal medium containing 20 g sucrose/l (= 8.42 mg C/ml). Unless otherwise stated, the cultures were incubated for 8 days at 24—26° C, after which the mycelia from each flask of each treatment was separately filtered, washed, dried in hot-air oven and finally weighed. The dry weight of the mycelium was taken as an index of growth.

For spore germination tests, the method used was the same as described earlier (Grover, 1964). Unwashed spores collected with the help of camel hair brush were used in all cases. Two dimensional paper chromatography was used for the determination of carbohydrates in the hydrolysates of mycelium and spores. The solvents used were n-butanol-acetic-acid-water (4 : 1 : 5 v/v/v) and 80% phenol, and the carbohydrates were detected by spraying the chromatograms with Partridge's ammonical silver nitrate (Block et al., 1958). The hydrolysates of spores and mycelium were prepared by the method described by Crossan and Lynch (1958). The identity of carbohydrates on chromatograms was compared with standard compounds on another chromatogram run simultaneously. Estimation of total residual carbohydrates in the culture filtrates was made by phenol-sulphuric acid method (Snell, Snell and Snell, 1961) using Carl Zeiss USU-1 (Jena) Spectrophotometer at 490 μ wave-length.

The chemicals used were of reagent grade supplied by B. D. H., London, or E. Merck & Co., Germany. All culture vessels used were of Pyrex glass.

The evaluation for spore colour and sporulation were subjective, but as far as possible all the data were subjected to statistical analysis.

Results

The Effect of Different Carbon Concentrations:

With sucrose as a carbon source, a continuous increase in growth was obtained uptil 12.5—15% concentration, after which it descreased gradually (Fig. 1). Residual carbohydrates in the filtrate began to appear when the initial sucrose concentration was 5.0% or more in the medium. Sporulation increased uptil a concentration of 2.0% sucrose, after which it became constant. The colour of the spores en masse changed from light olive green to green to deep green with the increase in sucrose concentration in the medium.

When the spores harvested from above cultures were germinated in distilled water, their germination was optimum (36.0%) when the spores were obtained from cultures having 30 ± 5 g/l of sucrose, while the spores obtained from media containing higher or lower sucrose concentrations gave poor germination (Fig. 1).

With glucose as carbon source, almost similar results were obtained. It was seen that with the increase in incubation period upto 20 days, a corresponding increase in mycelial dry weight was obtained at different glucose concentrations. But spores harvested from media containing 2.0% glucose and incubated for 8 days gave optimum spore germination as compared to other treatments.

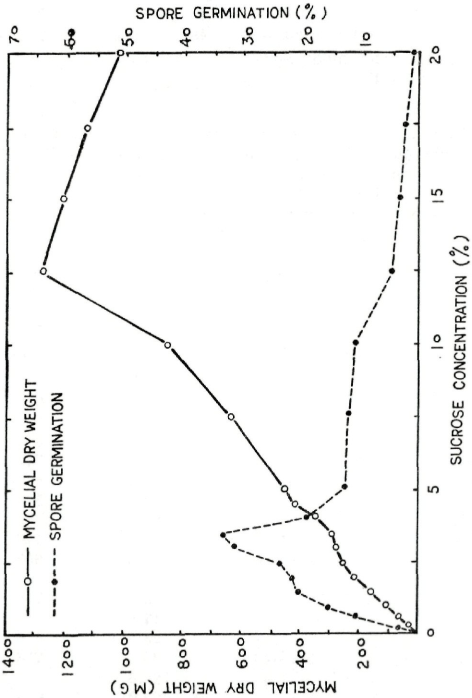

Fig. 1. Mycelial growth of *Aspergillus flavus* on different concentrations of sucrose and subsequent germination of spores in distilled water

The Effect of Different Carbohydrates:

Growth, Sporulation and Subsequent Spore Germination: *A. flavus* grew on all the 18 different carbohydrates when used individually as the sole carbon source in the basal medium. Presence of fructose, glucose, maltose, sucrose or raffinose as the sole carbon source in the basal medium increased the growth significantly, while lactose or sorbose yielded very poor growth (Table 1). Some increase in mycelial dry weigths were obtained in the latter cases when the cultures were incubated for longer durations of 16 days or more. In most other cases there was reduction in growth with the increase in the incubation period. Very little or no residual carbohydrates were seen after 8 days incubation period in the media containing ribose, xylose, arabinose, fructose, mannose, sucrose, cellobiose, melibiose, or melizitose; while abundant residues were found when galactose, lactose, or starch were the sole carbon sources in the media, though with the increase in incubation period, the residues decreased.

Table 1. Effect of various carbohydrates on the growth, sporulation and subsequent spore germination of *Aspergillus flavus*

Index of sporulation: + poor; ++ fair; +++ good; ++++ abundant. Each carbohydrate added at the rate of 8.42 mg C/ml. All observations as average of three replications

Carbon source	Sporulation	% spore germination	Mycelial dry wt. (mg)	Carbohydrate residue (mg C/ml)
D (—) ribose	++	21.3	437	0
L (+) rhamnose	+++	6.7	660	1.85
D (+) xylose	++++	32.2	642	0
L (+) arabinose	++++	31.4	509	0
D (—) glucose	++++	36.2	772	0.63
D (—) fructose	++++	6.2	861	0.12
D (—)galactose	++	7.9	565	4.0
D (+) mannose	++++	22.6	649	0
L (—) sorbose	+	15.1	176	0.87
Sucrose	++++	31.6	637	0
Lactose	+	23.9	66	3.6
Maltose	++++	5.0	768	1.1
Cellobiose	++++	17.8	633	0
Melibiose	++++	23.9	456	0
Raffinose	++++	33.7	756	0.98
Melizitose	+++	26.6	511	0
Inulin	++++	23.9	615	1.87
Starch	++++	15.8	688	2.5
L. S. D. at 5% level		5.6	38	

Most of the carbohydrates supported abundant sporulation. Poor sporulation was obtained on media containing lactose or sorbose, and fair sporulation was with ribose and galactose. Considerable variation was seen when the spores harvested from these media were germinated in distilled water. Spores harvested from media containing xylose, arabinose, glucose, sucrose, or raffinose gave normal germination (31.4—36.2%), while spores harvested from media containing fructose, galactose, maltose, or starch gave poor germination. Increase in age of spores reduced spore germination in most cases.

Spore Germination: Spores grown on the basal medium containing sucrose as the carbon source were germinated in each of the 18 different carbohydrates at 3 different concentrations. The spore germination was greatly influenced by the different carbohydrates when compared with the normal (germination in distilled water (32.6 ± 1.4%) (Table 2). Spore germination in the presence of ribose, glucose, fructose, or sucrose was significantly higher at all the concentrations, while no spore germination was obtained in the presence of cellobiose, raffinose, melizitose, maltose, or inulin. Very poor germination of spores was recorded in rhamnose, arabinose, or mannose. Increased concentration of carbohydrates increased spore germination but when fructose, galactose, sorbose or sucrose were used, the percentage germination decreased with the increase in their concentration. However, the 8.4 mg C/ml concentration of carbohydrates gave a satisfactory indication of these carbohydrates on germinability of spores of *A. flavus*.

Tab. 2. Germination of spores of *Aspergillus flavus* obtained from medium containing sucrose (8.42 mg C/ml) in different concentrations of carbohydrates

Carbon concentration as mg C/ml. Spore germination as average of three replicates. L. S. D. at 5% level = 8.6
Germination in distilled water = 32.6 ± 1.4%

Carbon source	Percentage spore germination at carbon concentration (mg C/ml)		
	4.2	8.4	21.0
D (—) ribose	27.1	48.9	66.0
L (+) rhamnose	5.3	6.4	8.9
D (+) xylose	11.1	14.3	35.5
L (+) arabinose	0	5.4	31.9
D (—) glucose	41.5	56.5	71.8
D (—) fructose	47.7	75.9	57.9
D (—) galactose	24.8	26.8	28.5
D (+) mannose	2.8	7.1	21.3
L (—) sorbose	25.7	36.8	28.4
Sucrose	24.5	54.6	22.6
Lactose	10.4	17.4	38.1
Maltose	0	10.4	26.9
Cellobiose	0	0	0
Melibiose	0	0	30.2
Raffinose	0	0	0
Melizitose	0	0	0
Inulin	0	0	0
Starch	0	16.5	35.5

Effect of Carbohydrates in Mixture

It was seen in the previous studies that six carbohydrates viz., galactose, glucose, maltose, fructose, sucrose and xylose were conspicuous for their role as good growth supporters or for increasing spore germination of *A. flavus* (Table 1 & 2). Besides, it was assumed that there might be a difference in the way a carbohydrate affected growth, sporulation and subsequent spore germination of the organism in the presence of other carbohydrates in the basal medium. To test this hypothesis these six carbohydrates were incorporated in equal proportions in the basal medium to give a total of 8.42 mg C/ml concentration. From these six carbohydrates in the basal medium one was omitted at a time, keeping the total carbon concentration the same, and the differences in the growth, sporulation and subsequent spore germination in different media were attributed to the missing carbohydrate.

It was observed that the absence of fructose from the basal medium did not affect the mycelial yield when compared with the medium containing all the six carbohydrates (Table 3). In other cases when one of the six carbohydrates were omitted from the mixture, the growth was slightly less than the complete medium and least growth being in case when sucrose was omitted.

The sporulation was not affected in any case. When spores were germinated in distilled water, significantly higher germination was obtained in those harvested from media devoid of xylose or maltose. While considerable decrease in spore germination was seen in case of those harvested from media devoid of fructose, glucose, sucrose or galactose. If the spores obtained from the latter 4 media were germinated separately in the respective carbohydrate that was lacking in the original medium the germination was restored to normal.

Effect of Substrate Carbon Sources on Spore Germination in different Carbon Compounds

Since the exogenous supply of carbohydrates greatly influenced the spore reserves, it was intended to see if there was any specific exogenous requirement of spores for increased germination. For this the spores obtained from media containing xylose, glucose, fructose, galactose, sucrose and maltose were germinated in distilled water and also in the above mentioned carbohydrates (8.42 mg C/ml).

As also pointed out earlier that spores harvested from media containing fructose, galactose or maltose germinated poorly in distilled water, but their germination was considerably increased when supplied with an exogenous carbon source (Table 4). Maltose when provided exogenously proved inhibitory to spore germination, irrespective of the spore source. On the other hand, exogenous supply of glucose and less so of sucrose increased spore germination when obtained from different sources.

Table 3. Dry weight of mycelium, sporulation and subsequent spore germination of *Aspergillus flavus* containing different combination of six carbohydrates

The complete medium consisted of the six carbohydrates, each in equal proportion to make a total of 8.42 mg C/ml in the medium

Medium	Sporulation	% spore germination	Mycelial dry weight (mg)
Complete	++++	30.7	624
Glucose omitted	++++	2.1	566
Fructose omitted	++++	12.4	620
Xylose omitted	++++	68.8	543
Galactose omitted	++++	13.2	532
Maltose omitted	++++	70.4	548
Sucrose omitted	++++	3.1	526
L. S. D. at 5% level		5.8	27

The highest germination induced by these chemicals was in case of spores obtained from the medium containing fructose and supplied exogenously with xylose, and those obtained from medium containing glucose and supplied with fructose or vice versa. Increased germination was also seen when spores were from medium containing xylose and germinated in xylose. No other similar case was noted.

Endogenous Carbon Sources in Spores

The reducing sugars present in the spores and mycelium of *A. flavus* when grown on media containing six different carbon sources either alone or in combination were tested chromatographically for their internal reserves. Results were evaluated qualitatively only and presented in Table 5.

It was seen that the spores obtained from media containing different carbon sources had in them both glucose and fructose, while maltose was present in addition to these in case of spores obtained from media containing fructose and maltose. Similarly, in the mycelium fructose was present in all cases and in addition to glucose and/or maltose were present in case of mycelia obtained from media containing glucose, fructose and sucrose. Xylose was also detected in mycelium from media containing sucrose, xylose and galactose.

Discussion

Carbon sources that favour abundant mycelial growth may or may not favour abundant sporulation and consequently the spores formed on media containing different carbohydrates germinated differently irrespective of the fact that a carbon source was good for growth and spore production. Fructose and maltose are the sugars which yielded maximum mycelial growth and sporulation in *Aspergillus flavus* but spores produced on these media germinated poorly. In contrast, the media containing glucose, sucrose or raffinose yielded abundant mycelial growth, sporulation and their spores germinated well. Obviously, the latter carbon sources contributed to suitable spore reserves which enabled these to germinate properly.

Perlman (1965) pointed out that most of the fungi, if not all, degrade carbon compounds added to the substrate medium and form vegetative and reproductive structures from the metabolic products. There is no positive report to show that the carbon sources added to the medium are incorporated directly into the mycelial carbohydrates, lipids or proteins. Our study shows that the incorporation of specific carbon sources in the medium definitely affects the spore reserves. Besides, the carbon residues left in the culture filtrates after the growth of *A. flavus* on media containing different carbon sources are not always related to mycelial growth, for, in case of media containing ribose, arabinose, melibiose, or melizitose no carbon residues are seen after 8 days growth of the organism and the mycelial growth by far is not abundant in these. Total utilization of carbon sources of diverse configuration may be indicative of the versitality and adaptive nature of the organism (Wilson and Lilly, 1958; Allen, 1965).

Table 4. Effect of substrate carbohydrates on spore germination of *Aspergillus flavus* in different carbohydrates
Cultures grown on medium containing carbohydrates at the rate of 8.42 mg C/ml
Germination of spores was in different carbohydrates having 8.42 mg C/ml concentration

Source of spores grown on media	percent spore germination in different carbohydrates						
	Water	Glucose	Fructose	Xylose	Maltose	Galactose	Sucrose
Xylose	14.3	39.1	26.3	24.4	0	24.8	29.2
Glucose	35.5	38.6	64.0	49.6	0	1.8	30.7
Fructose	6.8	56.4	17.0	58.9	2.7	20.1	25.9
Galactose	7.8	20.7	15.2	29.5	0	10.7	37.5
Sucrose	32.2	55.4	74.2	16.0	10.8	25.9	53.7
Maltose	5.0	41.5	43.2	53.0	0	0.6	41.2

Several carbon sources like fructose, maltose, glucose, sucrose or ribose when supplied exogenously to the spores produced on media containing sucrose increased their germination; others like rhamnose, arabinose, mannose or starch reduced the germination, while in the presence of cellobiose, melibiose, raffinose, melizitose or inulin there was complete inhibition of germination. The initiation of spore germination process results in the increase in the respiration and utilization of spore reserves (Cochrane et al., 1964; Allen, 1965). Eilers and Sussman (1964) reported that if a carbohydrate is added exogenously for the germination of *Neurospora tetrasperma* spores, its uptake begins only after 2 hours following activation. In other words, the exogenous supply of carbohydrates that increase spore germination act after spore activation in a complementary way in the metabolic process of spore germination. Cochrane et al. (1963) found that in case of chlamydospores of *Fusarium solani* germination of spores is accompanied by an increase in the oxidation of only those sugars which can be utilized for germination and sugars which support germination are oxidized more rapidly than those which do not. But Caltrider and Gottlieb (1966) are of the opinion that althuogh exogenously supplied carbohydrates are hydrolyzed during the process of smut spore germination, yet there is no relationship between oxygen consumption with carbon source and their effectiveness for germination. Lipids are thought to be acted upon first during the process of spore germination but there is now evidence that most spores require simultaneous utilization of lipids and carbohydrates (Sussman and Halvorson, 1966). Allen (1965) reported that the carbon source used for germination is taken up and converted to some temporary reserve and is later drawn upon again as the synthesizing capacity of spores increased. The inhibitory effect of certain carbohydrates, therefore, may be assumed to be due interference by these chemicals in the metabolic pathways leading to spore germination.

The fact that in a medium a mixture of six carbohydrates were essential for the growth, sporulation and normal subsequent spore germination is indicative of their specificity for these different processes. Sporuation and growth is not much affected if any of these carbohydrates are omitted from the substrate medium. Subsequent spore germination, however, is much affected, since for instance, ommission of maltose or xylose from the mixture in a medium produced spores which germinated better while whereas when any of the other carbohydrates are omitted in the medium the spores germinated poorly. In other words, presence of glucose, fructose, sucrose and galactose is essential for developing spores having normal reserves in them, whereas presence of maltose or xylose in a sugar mixture influences the spore reserve formation which inhibit their proper germination. Furthermore, the exogenous supply of maltose to the spores produced on media having different carbohydrates result in their germination inhibition, which is not the case with xylose. This suggest a different effect of maltose than that of xylose on *A. flavus*.

Although it is not expected that the spore reserves will include the carbohydrates on which these have been grown, yet it becomes apparent that the spore reserves contained glucose, fructose and/or maltose, irrespective of the substrate carbon source. Galactose is found in case of spores obtained from media containing galactose, or the mixture of six carbohydrates. The mycelia obtained from different media showed some variations in their carbohydrate contents. In case of conidia of *Erysiphe hordei,* Malca, Murray and Zscheille (1962) found free reducing sugars upto the extent of 1.5% of spore weight, while Edwards and Allen (1966) found large quantities of mannitol and less so of arabitol and other soluble sugars in the conidia. No sugar alcohols have been detected in the spores of *A. flavus.* The presence of free sugars like glucose, fructose, maltose or galactose in the spores of *A. flavus* in no case indicate that these are directly involved in the process of spore germination, though these could serve as good substrate materials for their respiration. It is clear that the exact nature of biochemical transformations undergone at the time of spore germination in relation to their reserves still need further investigations.

Summary

Aspergillus flavus utilized large amounts of sucrose (12.5—15.0%) for its optimum growth. The sporulation was optimum at 2.0% sucrose concentration, while the spores formed at media containing 3.0% sucrose germinated best. With glucose increased growth was obtained upto 20 days incubation period, though spores formed on 8th day germinated best. Among the 18 carbohydrates tried as individual carbon sources, best growth and sporulation was obtained on media containing fructose, glucose, raffinose and maltose, while least growth and sporulation was in media containing lactose and sorbose. No carbon residues were left in media containing ribose, xylose, arabinose, mannose, sucrose, cellobiose, melibiose or melizitose, while maximum residues were in case of galactose, lactose or starch. Absence of residue in the culture filtrates was not always related to good growth of the organism.

Spores harvested from media containing rhamnose, fructose, galactose, sorbose, maltose, cellobiose, inulin or starch germinated very poorly. The spores obtained from media containing sucrose when supplied with ribose, glucose, fructose, galactose, maltose or sucrose germinated better than in water, whereas in the presence of rhamnose, xylose, arabinose, mannose or lactose, the germination was poorer and there was no germination in cellobiose, melibiose, raffinose, melizitose or inulin. Presence of galactose, glucose, fructose or sucrose in a mixture gave good growth and sporulation and produced spores which germinated normally. Extreneous supply of glucose to spores produced on media containing different carbon sources increased their germination while maltose inhibited their germination. Spores obtained from media containing different carbohydrate sources had in their reserve glucose, fructose and/or maltose and galactose as identifiable carbohydrates, while the constituents of the mycelium varied greatly with the substrate carbon source.

Table 5. Endogenous carbohydrates in the spores and mycelium of *Aspergillus flavus* when grown on different carbohydrate substrates
All carbohydrates used at a concentration of 8.42 mg C/ml in the basal medium

Subtrate carbohydrate in the medium	Organ	Endogenous carbohydrates
Xylose	Mycelium	Glucose, fructose, maltose, galactose, xylose
	Spores	Glucose, fructose
Fructose	Mycelium	Glucose, fructose, maltose
	Spores	Glucose, fructose, maltose
Glucose	Mycelium	Glucose, fructose, maltose
	Spores	Glucose, fructose
Maltose	Mycelium	Fructose, maltose
	Spores	Glucose, fructose, maltose
Sucrose	Mycelium	Glucose, fructose, maltose, xylose
	Spores	Glucose, fructose
Galactose	Mycelium	Glucose, fructose, galactose, xylose
	Spores	Glucose, fructose, galactose
Mixture of 6 carbohydrates	Mycelium	Glucose, fructose, galactose
	Spores	Glucose, fructose, galactose, xylose

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