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Formation of Proteases by *Aspergillus fumigatus* and *Penicillium sp.*

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Abstract. Formation of proteases by *Aspergillus fumigatus* and *Penicillium sp.* under various environmental and nutritional conditions were investigated. A good amount of protease was produced by *A. fumigatus* when grown on glucose – peptone – gelatine medium, pH 5.0 at 30°C for four days. The same was found for a *Penicillium sp.* when grown on the same medium, pH 6.0 at 25°C for five days. Sucrose, ribose and raffinose stimulated the production of proteases by both fungi. The production of proteases by both fungi depends on C/N ratio in the medium. The maximum yield of proteases by *A. fumigatus* and *Penicillium sp.* in the broth medium was 5565.302 μg/ml and 30.746 μg/ml respectively.

Introduction

Many works have been done on proteases produced by fungi. Proteases produced by *Asperigillus sp.* have been investigated [1-12]. While proteases produced by *Penicillium sp.* were investigated [13,14], there is no information about proteases from *Aspergillus fumigatus*.

In Saudi Arabia studies on proteases from *Aspergillus* and *Penicillium* have been lacking. In our present work we have investigated the formation of proteases by *Aspergillus fumigatus* and *Penicillium sp.* isolated from Thoqbah soil and the cultural conditions that affect their production.

Materials and Methods

Medium

Glucose – peptone – gelatine medium (GPGe), 50 ml/250 ml capacity flask, was used [11]. It contained (g/L): K_2HPO_4 2.0; glucose, 1.0; peptone, 5.0; gelatin, 15.0; H_2O, 1L and pH 7.5, before sterilization.

Organisms

The proteolytic fungi *Aspergillus fumigatus* and *Penicillium sp.*, were previously isolated from Thoqbah soil in Saudi Arabia [11]. These two fungi were selected for further work on proteases, because they were the most potent, as determined by the size of zones of hydrolysis on G P Ge agar medium. Identifications were made according to Raper *et al* and Gilman [15,16].

Proteases assay

Gelatine clear zone cup agar plate method, GCZ [12] was used. In this method, 15.0 gm Difco agar and 15.0 gm Difco gelatine dissolved in one liter of phosphate buffer solution at pH 7.0, used to set plates. Three cups were made per plate; in each cup 0.1 ml of cell-free filtrate (crude filtrate) was transferred. After incubation for 6 hr. at 30°C, the plates were flooded with mercuric chloride solution, then mean diameters of the clear zones were measured to the nearest 0.5 mm. This method was referred as Gelatine Clear Zone method (GCZ).

Linearity of the standard curve that constructed before [12], between Merck pancreatic trypsin enzyme, concentrations, range from 10.870–25000 μg/ml, and mean clear zone diameters was used in assaying proteases as a reference (E.C. in μg/ml).

Formation of proteases under various cultural conditions

The various cultural conditions, on both liquid media and agar plates, used were: different incubation times (1–10 days), different temperatures (20–55°C), pH's, using sodium citrate and phosphate buffers covering pH ranges (4.0–8.0), different carbohydrates substituted glucose in GPGe medium as equal weights (0.5%), different nitrogen sources substituted peptone in GPGe medium as equal weights (0.5%), different protein sources substituted gelatine in GPGe medium as equal weights (1.5%) and different C/N ratio i.e. glucose/$NaNO_3$ ratio in Czapek's medium as equivalent C and N contents. All these different conditions are indicated in respective tables in the text.

In all experiments, the cell-free cultural filtrates (crude filtrate) were assayed for proteases activity using GCZ method. Mycelial dry weight from the liquid medium, M.D. wt. in mg/50 ml, linear growth diameter L.G.D. in mm, using inocula (8 mm in diameter), and clear zone diameters, C.Z.D. in mm around the growth on the agar plate medium were determined after flooding with mercuric chloride solution, to elucidate the relation between growth and proteases production. Enzyme concentrations, E.C. in μg/ml, were calibrated from the standard curve. In all experiments, the mean of triplicate readings was recorded.

In all Tables legend M.D. wt. = Mycelial Dry Weight in mg/50 ml liquid medium, E.C. = Enzyme Concentration in μg/ml calibrated from the standard curve, L.D.G. = Linear Diameter of Growth on agar plates in mm.

Results

As shown in Table 1 and Fig. 1 the high yields of proteases by *Aspergillus fumigatus* were noticed after four days and by *Penicillium sp.* after five days. The production of proteases by both fungi paralleled growth, but after 5 days the enzyme activity of the crude filtrate decreased.

Table 1. Production of proteases by *Aspergillus fumigatus* and *Penicillium sp.* at different incubation periods grown on GPGe broth with 50 ml in 250 ml flasks at 30°C.

Incubation period (day)	*Aspergillus fumigatus*				*Penicillium sp.*			
	M.D.wt. (mg)	E.C. (μg/ml)	L.D.G. (mm)	E.C. (μg/ml)	M.D.wt. (mg)	E.C. (μg/ml)	L.D.G. (mm)	E.C. (μg/ml)
1	10	17.263	12	48.828	10	17.263	10.3	21.740
2	30	17.263	15	69.053	20	17.263	15	69.053
3	50	21.740	20	173.920	50	24.414	20	347.839
4	60	30.746	25	390.625	100	34.527	23.5	2209.71
5	60	30.746	30	983.815	150	44.063	25	12500
6	100	24.414	35	5565.302	160	44.063	28	< 25000
7	130	24.414	40	< 25000	160	43.481	39	< 25000
8	130	24.414	45	< 25000	160	34.481	45	< 25000
9	130	21.740	50	< 25000	160	30.746	45	< 25000
10	120	21.740	50	< 25000	160	30.746	45	< 25000

The optimum temperature for proteases production by *Asp. fumigatus* was 30°C and by *Penicillium sp.* was 25°C as shown in Table 2. At 50 and 55°C on the solid medium, no growth of either fungus was noticed but a large amount of proteases was detected.

Table 3 shows that as pH increased the production of proteases increased by both fungi, using sodium acetate and phosphate buffer, till optimum pH for production by *Asp. fumigatus* (pH 5.0) and by *Penicillium sp.* (pH 6.0) was reached. Then the production of enzyme decreased till (pH 7.0). After that, the production increased. The amount of growth paralleled the enzyme production in the same manner.

As shown in Table 4, sucrose, ribose and raffinose were good carbohydrates for proteases production by *A. fumigatus* and *Penicillium sp.* Glucose supports good growth of *A. fumigatus,* but did not increase the production of the enzymes. Starch neither supports the growth nor increases the production of enzymes by *A. fumigatus.* Strach and glucose did not have any effect on *Penicillium sp.*, too.

Table 2. Production of proteases by *Aspergillus fumigatus* and *Penicillium sp.* grown on GPGe medium at different temperatures.

Temperature (°C)	*Aspergillus fumigatus*				*Penicillium sp.*			
	M.D.wt. (mg)	E.C. (μg/ml)	L.D.G. (mm)	E.C. (μg/ml)	M.D.wt. (mg)	E.C. (μg/ml)	L.D.G. (mm)	E.C. (μg/ml)
20	110	21.740	14	30.746	80	30.746	22	173.920
25	120	24.414	15	34.527	90	55.063	25	983.815
30	90	30.746	16	43.481	70	43.481	25	390.625
35	70	24.414	15.5	34.527	60	34.527	20	173.920
40	60	12.207	10	30.746	50	30.746	10	24.414
45	50	12.207	10	30.746	10	30.746	10	24.414
50	0.0	0.0	0.0	45.063	0.0	0.0	0.0	24.414
55	0.0	0.0	0.0	45.063	0.0	0.0	0.0	24.414

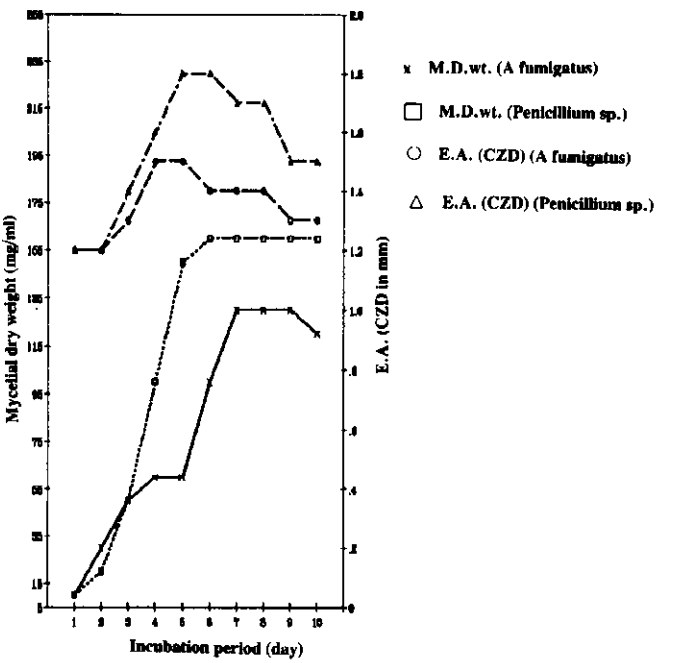

Fig. 1. Production of proteases by *Aspergillus fumigatus* and *Penicillium sp.* at different incubation periods at 30°C, in GPGe liquid medium M.D.wet = Mycelial Dry weight in mg/ml; E.A. = Enzyme Activity or crude filtrate by Clearing Zone Diameter in mm (CZD)

Table 3. Production of proteases by *Aspergillus fumigatus* and *Penicillium sp.* grown on GPGe medium at different pH's using sodium acetate and phosphate buffer.

pH	*Aspergillus fumigatus*				*Penicillium sp.*			
	M.D.wt. (mg)	E.C. (μg/ml)	L.D.G. (mm)	E.C. (μg/ml)	M.D.wt. (mg)	E.C. (μg/ml)	L.D.G. (mm)	E.C. (μg/ml)
4.0	90	12.207	15	69.053	110	17.263	30	659.662
4.6	100	69.053	25	983.815	120	21.740	36	1391.33
5.0	120	173.920	29	2209.71	140	30.745	60	<25000
5.6	130	97.656	30	5565.30	170	34.527	53.5	<25000
6.0	120	97.656	27	390.625	190	43.481	27	983.815
6.6	50	69.053	20	173.920	80	34.527	25	390.625
7.0	10	69.053	19	69.053	60	30.745	22.5	195.313
7.6	60	173.920	23	124.562	70	30.745	26	276.214
8.0	90	97.656	23	124.562	90	30.745	25	245.960

Table 4. Effect of different carbohydrates substituted glucose in GPGe medium (as equal weights) on the production of proteases by *Aspergillus fumigatus* and *Penicillium sp.*

Carbohydrate (0.5%)	*Aspergillus fumigatus*				*Penicillium sp.*			
	M.D.wt. (mg)	E.C. (μg/ml)	L.D.G. (mm)	E.C. (μg/ml)	M.D.wt. (mg)	E.C. (μg/ml)	L.D.G. (mm)	E.C. (μg/ml)
Without carbohydrate	140	34.527	15	695.662	60	17.263	22	3125
Glucose	290	122.980	15	34.527	80	17.263	20	2209.71
Sucrose	210	390.625	20	173.920	150	30.746	20	5565.30
Ribose	190	390.625	28	390.625	140	21.740	24	4425.07
Raffinose	220	245.960	20	195.313	120	21.740	20	2209.71
Starch	190	97.656	20	276.214	100	17.263	25	8838.83
Cellulose	110	17.263	15	48.828	180	17.263	20	5565.30

Table 5 shows that gelatine was a good nitrogen source for production of proteases by *A. fumigatus*, casien and peptone came next. It was shown from the results that the addition of any organic or inorganic nitrogen sources with gelatine to the medium stimulated the proteases production by *Aspergillus fumigatus*. In case of *Penicillium sp.*, peptone and meat extract stimulated the production of proteases. There is no relation between the amount of growth and the amount of enzymes produced by both fungi in presence of proteins. Asparagine did not stimulate the production of proteases by *Penicillium sp.*, but stimulated it by *A. fumigatus*.

Table 5. Effect of different nitrogen sources substituted peptone in GPGe medium (as equal weights) on the production of proteases by *Aspergillus fumigatus* and *Penicillium sp.*

Nitrogen source (0.5%)	*Aspergillus fumigatus*				*Penicillium sp.*			
	M.D.wt. (mg)	E.C. (μg/ml)	L.D.G. (mm)	E.C. (μg/ml)	M.D.wt. (mg)	E.C. (μg/ml)	L.D.G. (mm)	E.C. (μg/ml)
Gelatine	80	5565.302	53	> 25000	40	17.263	35	2265.30
Albumine	660	245.960	70	< 25000	70	17.263	35	2265.30
Casiene	600	983.815	52	< 25000	30	17.263	30	12500
Meat extract	100	173.920	39	< 25000	60	21.740	31	2265.30
peptone	150	390.625	40	< 25000	90	23.323	34	7870.53
Yeast extract	140	245.960	45	< 25000	70	17.263	30	12500
Asparagine	90	245.960	40	< 25000	110	15.373	14	2265.30
$NaNO_3$	100	245.960	52	< 25000	70	17.263	34	2265.30

In Table 6 proteins without gelatine in the medium decreased the growth and did not stimulate the production of the enzyme by *Aspergillus fumigatus*, but had no effect in case of *Penicillium sp.*

Table 7 shows that C/N ratio (13.19) in the presence both of gelatine in the medium gave high yields proteases by both fungi and supported good growth.

All results show that both fungi produced higher yields of proteases on the solid medium than in the liquid medium.

Table 6. Effect of different protein sources substituted gelatine in GPGe medium (as equal weights) on the production of proteases by *Aspergillus fumigatus* and *Penicillium sp.*

Protein source (1.5%)	*Aspergillus fumigatus*				*Penicillium sp.*			
	M.D.wt. (mg)	E.C. (μg/ml)	L.D.G. (mm)	E.C. (μg/ml)	M.D.wt. (mg)	E.C. (μg/ml)	L.D.G. (mm)	E.C. (μg/ml)
Albumine	10	69.052	27	12500	60	17.263	37	2265.30
Casiene	60	69.053	30	12500	80	17.263	35	2265.30
Meat extract	10	69.053	26	*	60	21.740	40	*
Peptone	10	173.920	19	*	70	17.263	35	*
Yeast extract	60	69.053	13	*	102	17.263	35	*

* : No ppt formed after flooding with $HgCl_2$ solution and no clear zone appeared.

Table 7. Production of proteases by *Aspergillus fumigatus* and *Penicillium sp.* at different C/N ratio (as equivalent C and N contents) in Czapek's medium + 1.5% gelatine.

C/N ratio	*Aspergillus fumigatus*				*Penicillium sp.*			
	M.D.wt. (mg)	E.C. (μg/ml)	L.D.G. (mm)	E.C. (μg/ml)	M.D.wt. (mg)	E.C. (μg/ml)	L.D.G. (mm)	E.C. (μg/ml)
Zero	40	69.053	2.5	5565	90	17.263	2.0	390.625
0.01	140	97.656	2.5	5565	90	21.740	2.0	390.625
2.57	190	138.107	3.5	12500	90	24.44	2.5	390.625
13.19	930	245.960	7.5	< 12500	210	30.747	2.5	390.625
38.27	251	69.053	3.5	2210	220	21.740	2.0	173.920

Discussion

The production of proteases by both fungi paralleled growth, this agreed with several findings [17,18,12]. The enzyme activity decreased after 5 days, may be due to some biproducts that inhibit the enzyme activity, this agreed with our findings with the proteases activity of actinomycetes [12]. At 50 and 55°C both fungi produced some proteases, but they could not grow at these temperatures. These temperatures might not have been suitable for their growth, however the enzymes remain active. Gaur *et al* [19] reported that the thermostability of protease producing strains could be due to their genetic adaptability to carry out their biological activity at higher temperature.

Using sodium acetate and phosphate buffers, (pH 5.0) was the optimum for proteases production by *Aspergillus fumigatus* and with *Penicillium sp.* was pH 5.6. After these two optimum pH's, the production by both fungi decreased at pH 6.6, then increased at pH 7.6. These results may be due to the presence of more than one enzyme of proteases synthesized by both fungi. These enzymes may be acidic or alkaline proteases. Cohen [4] reported that *Aspergillus nidulans* synthesizes four neutral or alkaline proteases. Glucose did not increase the production of proteases by *Aspergillus fumigatus* and *Penicillium sp.* This result is in harmony with the findings of Islam *et al* and Omkolthorum [18,12]. Sucrose, ribose and raffinose stimulated the production of proteases by *A. fumigatus* and *Penicillium sp.* William *et al* [20] reported that arabinose gave highest yields by *Bacillus polymyxa.* They also reported that hydrolyzed starch gave the highest amount of enzyme, but this did not agree with our findings.

A. fumigatus and *Penicillium sp.* produced inducible enzymes of proteases. This is in harmony with a few findings [4,21,12]. Prudlov *et al* [22] reported that the production of proteolytic enzymes by *Fusarium graminearum* and *Alternaria sp.* increased in the presence of 0.5% protein in the medium. This supports our findings

but in the presence of protein with gelatine. Asparagine did not stimulate the production of proteases by *Penicillium sp*. This agrees with the findings of Prudov *et al* [22]. At the same time we found that asparagine stimulated the production of proteases by *A. fumigatus*. It has also been reported [3,12] that the production of proteases in cultural broth depended on C/N ratio in the medium; our findings support this.

The amount of protease yielded by *Aspergillus fumigatus* was higher (5565.302 μg/ml) than that by *Penicillium sp*. (30.746 μg/ml). Further work on partial purified proteases by these fungi is in progress.

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إنتاج أنزيم البروتيز بوساطة فطر اسبرجلس فيوميجاتس ونوع من فطر البنيسيليوم

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ملخص البحث. درس إنتاج أنزيم البروتيز بوساطة فطر اسبرجلس فيوميجاتس ونوع من فطر البنيسيليوم تحت ظروف بيئية وغذائية مختلفة. أنتج فطر اسبرجلس فيوميجاتس كمية جيدة من البروتيز عندما نمى على بيئة جلوكوز ـ ببتون ـ جيلاتين عند درجة تركيز أيون الهيدروجين ٥ عند درجة ٣٠°م لمدة ٤ أيام وأنتج فطر البنيسيليوم البروتيز عندما نمى على البيئة نفسها عند درجة تركيز أيون الهيدروجين ٦ عند ٢٥°م لمدة ٥ أيام.

وجد أن سكر السكروز والرايبوز والرافينوز ينشط إنتاج البروتيز بوساطة كلا الفطرين. وإنتاج البروتيز بوساطة كلا الفطرين يعتمد على نسبة الكربون إلى النيتروجين في البيئة.

إن أقصى إنتاج من البروتيز بوساطة فطر اسبرجلس فيوميجاتس وفطر البنيسيليوم في البيئة السائلة هو ٥٥٦٥,٣٠٢ ميكرو جرام لكل ملي و ٣٠,٧٤٦ ميكرو جرام لكل ملي على التوالي.