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SUBMERGED FERMENTATION OF AMYLASE ENZYME BY *ASPERGILLUS FLAVUS* USING *COCOS NUCIFERA* MEAL

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ABSTRACT
Soil samples were collected from the coastal region of Neendakara, along the West cost of Kerala, India. 15 fungal species were isolated and identified by using lacto phenol cotton blue staining method. From this, *Aspergillus flavus* was tested in Starch Hydrolysis Agar Medium for its amylase enzyme production under sumerged aerobic fermentation with different physico- chemical properties of substrates. *Cocos nucifera* meal was used as a carbon source. Heavy metals were added to these medium and were used as a modified medium. The effect of different carbon source, nitrogen compound and physico-chemical conditions like temperature, pH and incubation periods were studied for derivation of amylase enzyme. The molecular weight of enzyme was determined by SDS –PAGE. The role of heavy metals was determined by ion exchange chromatography. *Cocos nucifera* meal medium with dextrose has shown the highest amylase production at pH 6.0 and temperature of 30ºC with protein content of 201µg/ml; 98.4µg/ml and dry biomass of 1.28µg/ml

Keywords: Soil samples, *A. flavus*, amylase, SDS-PAGE, Ion exchange chromatography, submerged aerobic fermentation

INTRODUCTION
Enzymes are protein catalysts synthesized by living systems and are important in synthetic as well as degradative process. Amylases are enzymes that break down starch or glycogen. It is produced by a variety of living organisms ranging from bacteria, fungi to plants and humans (Pandey *et al*., 2000). Alpha amylase (endo-1,4-Dglucose- D glucohydrolase 3.2.1.1.) belongs to the family of endo amylases that randomly cleave the 1,4 – D glycoside linkage between adjacent glucose units in the product chain retaining the a- anomeric configuration in the product (Arun Sasi *et al*, Pandey *et al*., 2001).It is therefore important to increase protein utilizing by all ways and means the increasing world demand for food and feed protein led to search for a non-conventional protein source to conventional protein source. A great deal of interest has been focused on the potential of converting protein from agricultural waste like *Cocos nucifera* meal to microbial protein or single cell protein (Ravinder Rudravaram *et al*., 2004, Cushoma *et al*., 2005). The different meals were used as

fermentation medium (Hirokilshide *et al*., 2004, Oshome *et al*., 2005, Heidelbergh *et al*., 2006). In the present study the effect of different physic-chemical parameters, effect of various nitrogen sources, carbon source, heavy metals were also studied.

Oil cakes are the by products obtained after oil extraction. Depending upon the extraction methods, the chemical composition of oil cakes varies, they are fairly rich in protein and it was used in both feed ingredients for farm animals and agricultural feds (Singh *et al*., 2003). It has been used in the area of enzyme fermentation for utilization of raw materials for the production of value added fine products (Pandey *et al*., 2000). Oil cakes are rich in fibre and high concentration of non starch polysaccharide. Cocos nut meal contains 14-20% of crude protein.

Amylase are mainly produced by *Aspergillus* sp and *Trichoderma* sp. *Aspergillus oryzae* were selected for further studies. Molecular weight was determined by SDS-PAGE. Role of heavy metals were determined by Ion exchange chromatography, paper chromatography, paper chromatography, thin layer chromatography.

MATERIALS AND METHODS

Soil samples were collected from the coastal regions of Needakara , Kollam, along the west coast of Kerala, India. The soil samples were collected in a sterile container and brought to the laboratory for further processing. Soil samples were serially diluted up to 10-3 dilutions, then poured into sterile rose Bengal agar plates (RBA) and incubated at room temperature for 72 hrs (Gillman, 1998).

Microorganisms

In the present study15 fungal species were enumerated to include *A. fumigatus, A. oryzae, A.niger, A. flavus, A. nodulance, A. sulphurus, A. terreus, Trichoderma vessei, T. viridae, Penicillium citrinum, P. oxalicum, Fusarium moniliformis, F.oxalicum, F. oxysporum, Rhizopus oryzae* were isolated and identified by using lactophenol cotton blue staining method.(Kohlmeyer.J and Kohlmeyer, *et al*., 1979; Deschenamp F and Mc Huent,1985; Haltrich.P *et al*.,1996; Gillman, 1998 and Ainsworth,1968)

Screening for amylase producing fungi

The isolated strain was streaked into starch agar plate and incubated at room temperature for 72 hours. After incubation 1% of iodine solution was layered on the agar plates and zone of clearance was observed for screening the fungi (Pandey *et al*., 2006).

Submerged fermentation of Amylase

Submerged fermentation was carried out in the Ehlenmeyer flasks by taking 100 ml of amylase production medium (Bernfed, 1951); containing Peptone (6.0g/L), MgSO4 (0.5g/L), KCl (0.5g/L), Starch (1g/L). In addition to this certain agricultural waste products like Cocos nut meal (Cocos nut oil cake) were used as a submerged fermentation medium. Cocos nut meal was procured from local market in Pudukkottai, Tamil Nadu, India. 15 gm of oilcake was powdered, dry cake was taken. It was modified with certain carbon source, nitrogen source and heavy metals. Carbon sources (each 2g/L) included are glucose, fructose, mannitol, mannose, starch,

sucrose, lactose and dextrose. Nitrogen sources included are NH_4Cl, $NaNO_3$, NH_4NO_3, KNO_3, Peptone, Urea and Yeast extract (2g/L) as nitrogen source. Certain heavy (each 1mm/L) metals like Ca^{2+}, Cu^{2+}, Mg^{2+}, Fe^{2+}, Hg^{2+}, Zn^{2+} Mn^{2+}, K^{2+} and P^{2+} were also included in the production medium. The medium was maintained at a pH range of 3, 6 and 9, at 30°C on a shaker with 120rpm for 6 to 18 days (Heidelbergh and Springer Berlin 2006, Pandey *et al*., 1999).

Enzyme extraction

Crude enzyme was extracted by mixing a known quantity of fermented substrate with distilled water containing 0.1%. Tween 80 on rotator shaker at 180 rpm/1 hr. The suspension was then centrifuged at 7000xg at 4°C and the supernatant was used for enzyme assay (Pandey *et al*., 2006)

α- amylase assay

α-amylase activity was determined (Pandey *et al*., 1999). Then reaction mixture containing 1.25 ml of 1% soluble starch, 0.25ml of 0.1 mM acetate buffer (pH 5.0) and 0.25ml of crude enzyme extract was incubated for 10 minutes at 50°C. After incubation the reducing sugar was estimated by Dinitrosalicylic acid (DNS) method (Miller *et al*.,1959). For the estimation of enzyme ready colour developed reaction mixture was taken at 575nm using a Shimazhu-UV-1604 Spectrophotometer with glucose standard.

Estimation of soluble Protein

Soluble protein concentration was determined in aqueous extract fermented substrate using Bovine serum as standard (Lowrey *et al*., 1951).

Molecular mass determination

The molecular weight was determined by SDS-PAGE.(Sadasivam and Manikkam, 1997).

Dry Biomass detection

After incubation the growth medium was pasteurized at 65°C for 30 mins in a water bath. After each fermentation period, mycelia were removed from the flasks by passing through a dried and pre-weighed Whatman no.1 filter paper and washed twice with sterile distilled water . The filter paper was dried at 90°C -100°C by using hot air oven. Then dry weight was obtained (Haltrich.*et al*., 1996).

Ion exchange chromatography

In Ion exchange chromatography concentrated enzyme (2ml) was loaded onto an anion exchange DEAE Sepharose FF (Sigma- Aldrich Co, USA) column (15 nm diameter and height 100nm) at a flow rate of 0.5ml/min. Equilibration and elution were performed first with 0.05M Na- phosphate buffer to remove unbound proteins and then with a liner salt gradient from 0 to 3 M NaCl. Fraction (2ml) were collected and analyzed for amylase activity of protein content . Active fractions were pooled and concentrated and then purified using Cation Exchange CM Sepharose FF Sigma-Aldrich Co, USA as above. The active fractions were pooled (Steven *et al*., 1977).

RESULTS AND DISCUSSION

Soil samples were collected from the coastal area of Neendakara, Kollam district, Kerala, India (Fig: 2). In the present study 15 fungal species were enumerated namely *A. fumigatus, A. oryzae, A. niger, A. flavus, A.nodulance, A.sulphurus, A. terreus, Trichoderma vessei, T.viridae, Penicillium citrinum, P.oxalicum, Fusarium moniliformis, F. Oxalicum, F. oxysporum* and were identified by Lactophenol cotton blue staining method.(Kohlmeyer.J and Kohlmeyer, *et al*., 1979; Deschenamp F and Mc Huent,1985; Haltrich.P *et al*.,1996; Gillman, 1998 and Ainsworth,1968). (Fig:1 Fig:3). From this *A. flavus* was selected for further studies (Fig.3). Among this *A.flavus* has shown apparently clear zone around the colonies when subjected to starch hydrolysis medium .Carbon and Nitrogen sources were altered and modified minimal medium (MMM) was prepared. MM7 has shown highest enzyme and protein activity of 162.2µg/ml and 68.62µg/ml respectively with a temperature of 30°C and pH 6 with an incubation period of 18 days (Table::1). But in case of MM4, MM3, MM6, MM2, MM1 have the capacity to produce enzyme activity 144.62µg/ml,118.2µg/ml, 110.2µg/ml, 54.162µg/ml, 49.12µg/ml, respectively. But incase of protein activity the protein content was 31.22µg/ml, 28.22µg/ml, 26.22µg/ml, 16.22µg/ml, 22.42µg/ml, respectively. Dry biomass of all the production media in crude form was 1.102µg/ml, 1.022µg/ml, 0.922µg/ml, 0.902µg/ml, 0.842µg/ml, 0.842µg/ml and 0.942µg/ml, respectively (Table: 2). But incase of carbon source, MM10 has shown high production at a rate of 86.42µg/ml and 30.42µg/ml, at 30°C and Ph 6. But in case of all other media MM14, MM1, MM12, MM13, MM15, MM9, MM8 (Table: 2) the enzyme activity was found to be 89.2µg/ml, 86.4µg/ml, 82.22µg/ml, 80.32µg/ml, 63.42µg/ml and 48.82µg/ml, respectively with a protein content of 26.42µg/ml, 26.2µg/ml, 23.4µg/ml, 21.4µg/ml, 20.2µg/ml, 19.2µg/ml, and 18.4 µg/ml, respectively. The role of heavy metals in enzyme production is crucial. Fe^{2+} has shown the highest enzyme activity 124µg/ml, than all the other metal ions. But Hg2+(32µg/ml,) inhibited the enzyme production.

Agricultural waste products like *Cocos nucifera* meal (Cocos nut oil cake) were used as a crude substrate ingredient in the fermentation medium to make *Cocos nucifera* meal medium (CNMM)(Fig.4) and the carbon and nitrogen source were altered to find out the rate of production of amylase. CMM3 has shown a higher enzyme activity (170.3µg/ml) and protein content (46.2µg/ml).(Fig.4)

In case of all the media CMM4, CM7, CMM5, CMM1, CMM2, CMM6 (Table:-3) the enzyme activity was found to be 163.1µg/ml, 161.2µg/ml, 160.3µg/ml, 157.3µg/ml and 152.2µg/ml, respectively and the protein activity was found to be 34.1µg/ml, 33.1µg/ml, 32.8µg/ml, 32.2µg/ml, 30.2µg/ml and 28.2µg/ml with corresponding dry biomass of 1.03µg/ml, 0.92µg/ml, 0.91µg/ml, 0.91µg/ml, 0.90µg/ml, 0.89µg/ml and 0.84µg/ml, respectively. In case of carbon source altered media, CMM10 was found to have highest enzyme activity (201.0µg/ml) with highest protein activity (98.4µg/ml). The enzyme activity of all the other media CMM9, CMM14, CMM13, CMM8, CMM15, CMM11 and CMM12 were found to be 190.4µg/ml, 188.4µg/ml, 181.2µg/ml, 180.4µg/ml, 176.2µg/ml, 128.2µg/ml and 96.8µg/ml respectively, with their corresponding protein content of 60.8µg/ml,

52.2µg/ml, 48.6µg/ml, 36.4µg/ml, 32.3µg/ml, and 30.1µg/ml, respectively with dry biomass 1.28µg/ml, 0.94µg/ml, 1.08µg/ml, 1.01µg/ml, 0.92µg/ml, 0.92µg/ml and 0.96µg/ml and 0.84µg/ml (Table:-4). In case of heavy metals Fe^{2+} has shown the highest enzyme activity of 132µg/ml. But Hg^{2+} was found to inhibit the enzyme activity (42µg/ml) (Table: 5). The molecular weight was determined by SDS-PAGE as13000KDa with Bovine Serum Albumin as standard protein (Fig 6). The high peak in the Ion Exchange Chromatogram indicated the role of metal ions in the production medium (Fig.7).

The present study has shown that *A. flavus* produced more amylase and soluble protein under submerged aerobic fermentation by using *Cocos nucifera* meal as a substrate in the production medium, dextrose as a carbon source, at pH 7, temperature 30°C, with an incubation period of 18 days. As the *Cocos nucifera* meal is economically cheaper than several laboratory chemicals, *Cocos nucifera* meal medium can be recommended as a production medium for large scale and Industrial production of amylase.

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Table:-1 Amylase production (µg/ml) by *A. oryzae* on minimal medium fermented with additional carbon source (2 µg/ml) with temp 30°C and pH 3, 6 and 9

Medium	Nitrogen source	Incubation period	pH enzyme activity (µg/ml)			pH protein activity (µg/ml)			Biomass (µg/ml)
			3	6	9	3	6	9	
MM1	NH_4Cl	6 12 18	22.12 34.08 42.08	34.10 42.03 49.21	21.12 28.10 32.18	3.8 10.4 16.8	14.4 18.7 22.4	8.2 6.4 30.12	0.82 0.84 0.94
MM2	$NaNO_3$	6 12 18	26.12 39.12 18.12	44.16 49.18 54.16	12.12 16.18 30.2	6.3 8.4 10.6	12.2 13.4 16.2	8.1 5.4 10.2	0.62 0.72 0.84
MM3	NH_4NO_3	6 12 18	32.16 42.18 90.8	44.2 54.4 118.7	21.23 32.2 82.1	12.1 13.2 14.1	13.4 16.2 28.2	12.1 13.1 14.6	0.82 0.84 0.90
MM4	KNO_3	6 12 18	32.18 44.18 98.16	49.2 72.1 144.8	30.1 42.12 71.2	6.2 12.4 21.2	13.2 14.8 31.2	16.2 10.2 14.2	0.83 0.84 0.92
MM5	Peptone	6 12 18	42.1 62.16 118.2	58.1 98.2 152.2	32.2 40.2 82.1	18.4 22.4 42.2	22.3 32.2 52.1	10.1 12.4 18.6	0.82 0.84 1.02
MM6	Urea	6 12 18	16.2 32.2 49.2	32.2 52.2 110.2	12.4 22.6 42.2	10.4 12.2 18.2	16.2 22.2 26.2	6.2 11.2 16.2	0.72 0.82 0.84
MM7	Yeast extract	6 12 18	41.2 61.2 120.2	63.2 98.2 162.2	32.1 52.1 108.4	16.2 20.2 30.2	18.2 26.2 68.6	8.2 16.2 20.1	0.72 0.84 1.10

Table:-2 Amylase production (µg/ml) by *A. oryzae* on minimal medium fermented with additional nitrogen source (2 µg/ml) with temp 30°C and pH 3,6 and 9

Medium	Carbon source	Incubation period	pH enzyme activity (µg/ml)			pH protein activity (µg/ml)			Biomass (µg/ml)
			3	6	9	3	6	9	
MM8	Glucose	6 12 18	8.2 12.18 18.16	26.2 38.2 48.8	6.1 10.4 26.2	3.2 6.7 10.2	6.8 16.2 18.4	2.8 4.2 10.6	0.76 0.73 0.82
MM9	Fructose	6 12 18	12.14 14.16 19.20	24.6 40.8 63.4	18.2 22.1 36.2	3.4 10.2 12.4	10.2 16.4 19.2	1.8 4.8 8.6	0.72 0.75 0.80
MM10	Dextrose	6 12 18	15.16 18.16 26.18	36.8 64.6 90.5	12.8 18.6 39.2	6.8 10.4 12.8	20.2 24.6 30.4	1.8 12.2 14.4	0.80 0.73 0.83
MM11	Lactose	6 12 18	11.6 18.8 22.10	34.2 60.3 86.4	10.6 20.4 30.2	3.2 10.6 14.2	6.8 16.4 26.2	4.2 9.6 12.4	0.72 0.76 0.82
MM12	Mannitol	6 12 18	10.8 16.8 20.8	24.2 56.2 82.2	10.4 16.9 26.2	1.5 6.2 10.2	11.2 16.4 23.4	3.4 6.8 14.2	0.71 0.73 0.80
MM13	Mannose	6 12 18	8.7 17.2 21.6	26.2 46.2 80.3	16.2 15.7 20.1	1.4 8.2 10.6	8.4 18.2 21.4	6.2 12.4 16.2	0.77 0.78 0.82
MM14	Starch	6 12 18	6.8 18.8 28.2	30.2 43.4 89.2	12.4 14.2 20.8	1.5 8.4 13.4	13.2 16.8 26.4	10.4 12.2 16.2	0.72 0.78 0.82
MM15	Sucrose	6 12 18	5.2 11.6 18.5	28.6 40.2 80.2	11.2 15.2 22.2	2.8 4.6 14.2	10.2 16.8 20.2	6.2 13.2 14.3	0.70 0.71 0.80

Table:-3 Amylase production (µg/ml) by *A.oryzae* on *Cocos*nut mealmedium fermented with additional nitrogen source (2 µg/ml) with temp 30°C and pH 3,6 and 9

Medium	Nitrogen source	Incubation period	pH enzyme activity (µg/ml)			pH protein activity (µg/ml)			Biomass (µg/ml)
			3	6	9	3	6	9	
CMM1	NH_4Cl	6 12 18	16.2 48.7 100.9	40.6 80.8 160.2	12.1 32.4 90.8	8.2 12.4 20.8	12.4 28.6 32.2	13.4 20.4 21.4	0.82 0.81 0.90
CMM2	$NaNO_3$	6 12 18	15.4 58.6 100.5	44.7 84.2 157.3	14.1 22.4 80.6	10.6 14.2 22.6	12.4 22.8 30.2	10.6 18.2 20.4	0.83 0.82 0……
CMM3	NH_4NO_3	6 12 18	25.2 47.8 98.7	48.7 90.2 170.3	14.4 24.2 82.1	10.5 16.8 28.4	22.2 38.6 46.2	18.2 21.4 28.1	0.83 0.81 1.03
CMM4	KNO_3	6 12 18	24.6 42.6 70.6	43.2 82.4 163.1	13.6 20.2 81.2	12.1 18.1 22.1	16.1 28.1 34.1	10.8 14.8 20.1	0.76 0.82 0.92
CMM5	Peptone	6 12 18	22.5 38.6 80.2	38.6 81.2 160.3	12.4 122.1 80.2	16.1 20.2 28.1	10.6 22.1 32.8	6.2 12.3 18.4	0.72 0.84 0.91
CMM6	Urea	6 12 18	16.8 32.6 78.4	36.4 70.2 152.2	14.2 31.2 81.2	6.2 12.4 22.1	10.3 19.2 28.2	10.2 18.2 20.1	0.71 0.86 0.84
CMM7	Yeast extract	6 12 18	22.4 38.6 90.2	30.2 89.4 161.2	12.2 89.3 74.6	18.1 20.2 24.1	19.1 24.1 33.1	10.4 14.8 28.1	0.77 0.84 0.91

Table:-4 Amylase production (µg/ml) by *A.oryzae* on *Cocos* nut meal medium supplemented with additional nitrogen source (2 µg/ml)

Medium	Carbon source	Incubation period	pH enzyme activity (µg/ml)			pH protein activity (µg/ml)			Biomass (µg/ml)
			3	6	9	3	6	9	
CMM8	Glucose	6	28.4	38.8	22.4	10.6	18.2	10.4	0.82
		12	46.2	60.2	46.4	18.6	28.6	16.2	0.83
		18	90.4	180.4	109.4	27.4	36.4	30.2	0.92
CMM9	Fructose	6	22.4	39.2	26.8	16.8	20.3	16.3	0.83
		12	44.6	63.7	44.5	20.3	32.4	20.4	0.76
		18	84.6	190.4	110.2	27.5	60.8	34.2	0.94
CMM10	Dextrose	6	39.4	90.4	38.6	14.8	42.3	26.2	0.98
		12	44.2	102.3	66.8	22.4	62.1	34.2	0.96
		18	134.6	201.1	184.8	40.8	98.4	42.1	1.28
CMM11	Lactose	6	24.2	34.5	32.1	10.6	16.4	14.2	0.92
		12	46.2	55.6	43.4	18.8	22.4	20.2	0.93
		18	70.63	128.2	76.8	26.4	32.2	32.2	0.96
CMM12	Mannitol	6	16.8	42.4	40.2	11.2	20.2	16.2	0.83
		12	43.4	52.6	18.6	16.2	28.8	18.4	0.83
		18	76.2	96.8	110.2	22.4	30.1	20.6	0.84
CMM13	Mannose	6	42.2	46.8	42.1	10.8	22.4	10.5	0.92
		12	64.2	68.6	62.2	16.8	32.4	30.2	0.94
		18	100.8	181.2	100.2	22.2	48.6	40.2	1.01
CMM14	Starch	6	42.6	44.2	40.2	22.6	32.4	16.8	0.94
		12	72.1	90.8	80.2	38.6	40.4	22.6	0.98
		18	124.6	188.4	112.4	40.5	52.2	34.2	1.08
CMM15	Sucrose	6	34.6	38.2	32.4	16.2	10.6	8.4	0.84
		12	73.2	74.3	42.4	18.4	30.2	16.8	0.86
		18	110.4	176.2	68.8	20.6	32.3	22.4	0.92

Table:-5. Effect of metal ions on the activity of amylase enzyme produced by *A. oryzae* on *Cocos nucifera* meal and minimal medium.

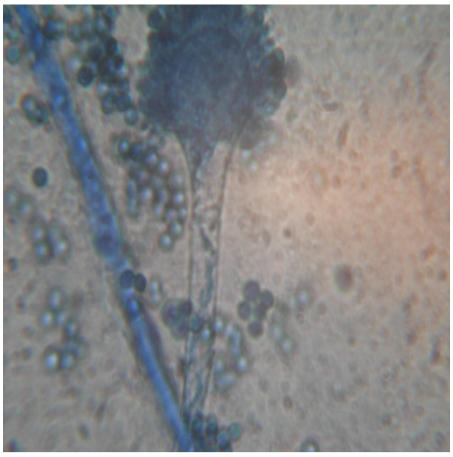
Medium	Metal ions (1gm)	Residual %
Cocos nucifera meal medium	None	100
	Ca^{2+}	112
	Cu^{2+}	124
	Mg^{2+}	128
	Fe^{2+}	132
	Hg^{2+}	42
	Zn^{2+}	118
	Mn^{2+}	116
	K^{2+}	122
	P^{2+}	127
MM	None	100
	Ca^{2+}	110
	Cu^{2+}	121
	Mg^{2+}	118
	Fe^{2+}	124
	Hg^{2+}	32
	Zn^{2+}	115
	Mn^{2+}	110
	K^{2+}	113
	P^{2+}	114

1) Plate shows culture of *A. flavus*

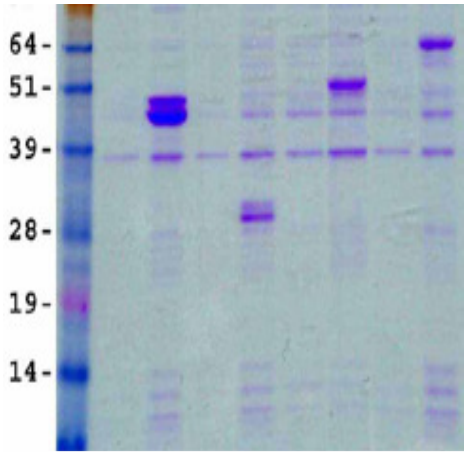
2) Collected soil samples

2) Mixed culture of fungi

4) Submerged fermentation medium

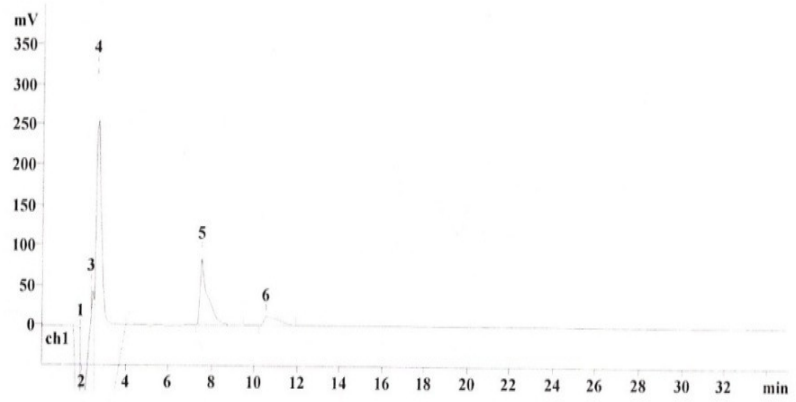


4) Microscopic view of *A.flavus*



5) SDS-PHAGE

SAMPLE-2



Sample Id	Element	Retention Time	Area	Concentration of inject	Dilution
Std	Na	7.61	810	10 ppm	
Std	K	11.06	434	10 ppm	
Nafeel-2	Na	7.47	2058.785	2540 ppm	50ml/0.5ml
Nafeel-2	K	10.49	546.299	1258 ppm	50ml/0.5ml

Ca & Mg are below detection limit

6)Ion exchange chromatography