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PRODUCTION OF AMYLASE ENZYMES BY FILAMENTOUS FUNGI

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ABSTRACT

Fifty-two out of one hundred and forty-four isolates of filamentous fungi were recorded as amylase producer, but with different degrees, on solid plate method. Chemical constituents of potato waste samples (collected from the Chpis' Factory for Food Industries, Assiut, Egypt) were determined by chemical analysis. Twelve isolates (eight highly producer isolates and four isolates isolated from potato wastes) were screened for amylase production on potato wastes. Aspergillus flavus443 was recorded as the best enzyme producer on potato wastes and synthetic medium. The best environmental and nutritional conditions for amylase production by Aspergillus flavus443 were : [50 gm of potato wastes with 10 ml distilled water , manitol (1%) as a carbon source , casein (1%) as a nitrogen source , pH 5 and the medium was incubated at 40 ºC for three days).

INTRODUCTION

Amylases are important extracellular enzymes ; stabile over a wide range of pH values and thermostabile enzymes employed in the starch processing industries for the hydrolysis of starch into simple fermented sugar (glucose) (Akpan *et al.*, 1999).

During the last decade, an increased attention was paid to the use of various agro- industrial wastes in solid-state fermentation (SSF) by filamentous fungi (Pandey & Soccol, 2000 and Pandey *et al.*, 1999; 2000a; 2001).It has been reported that SSF is the most appropriate process in developing countries due to the advantages it offers (Carrizales & Jappe, 1986; Alva *et al.*, 2007).

Fungal amylases are find potential application in a number of industrial processes such as in food, baking, brewing, detergent, textile pharmaceutical, confectionaries, paper, skin production, fruit processing and juice production, tea, coffee and chocolate syrups industries. With the advent of new frontiers in biotechnology, the spectrum of amylase application has expanded into many other fields, such as clinical, medical and analytical chemistry (Forgarty, 1983; Ibukun and Akindumila, 1998; Achi & Njoku, 1992; Okolo *et al.*, 1995 and Pandey *et al.*, 2000a&b). The pretreatment of the substrates from several fungi amylases were widely used in industry for production numerous products such as lipid production (Kaur and Worgan, 1982), organic acid like lactic acid (Huang *et al.*, 2003& 2005; Jin *et al.*, 2003), production of fungal protein and amino acids (Jin *et al.*, 1999), Ethanol

(Gregg & Sadder, 1995 and BBI, 2002) and biodeterioration of industrial paper (Rojas *et al.*, 2008).

A cost reduction in amylase enzyme production can be achieved by using less expensive substrates, such as agro-industrial waste products (Hang and Woodams, 1984, 1985, 1987; Aravantinos-Zafiris *et al.*, 1994; Khare *et al.*, 1995; Pandey *et al.*, 2000b; Vandenberghe, 2000a&b; Soccol *et al.*, 2003).

Potato tubers are abundant material that mainly contains fresh matter [moisture (80 ± 2%), starch (18 ± 2%), cellulose and hemicelluloses (1.5 ± 0.5%), glucose (0.4 ± 0.3%) and proteins (2 ± 1.5%) (Delgado *et al.*, 2009).

Annual world production of Potato is around 300 million tons, and areas planted cover more than 18 million ha. Major producing countries and the world's share of production are (China, 20%; Russia, 12%; India, 8% and United States, 8%) Delgado *et al.*, (2009) . Potato is one of the most important crops grown in Egypt for local consumption, export and processing. The area cultivated with potatoes about 212,000 acres producing about 2.2 million tons, with an average of 10.5 tons per acre (Hegazy, 2009). Biotechnology industries demand potato (*Solanum tuberosum*) the best raw materials to prepare growth media for the fermentative processes (Liu & Xu, 2008 and Delgado *et al.*, 2009).

The present study is aimed to screening of numerous Egyptian fungal isolates (144) for amylase production on synthetic medium. Also, utilization; optimization and maximization of both nutrition and environmental condition affecting amylase production on potato wastes by the most highly producer isolates were studied.

MATERIALS AND METHODS

I-Primary Screening of Isolates for Amylase Production

1- Collection of different fungal isolates

One hundred and forty–four isolates of filamentous fungi belonging to twenty two genera and fifty- five species in addition to two species varieties were tested for amylase production. These isolates were obtained from Botany Department, Faculty of Science, Assiut University, Egypt and AUMC (Assiut University Mycological Center). These isolates were isolated from different sources.

The isolates were maintained on slopes of Czapek's Dox agar medium (Smith & Onions, 1983a&b). Inoculums were prepared from a 7 day old culture in spore suspensions in 0.2 % (V\V) aqueous Tween 80.The pH was adjusted to 6.5 and incubated at 28 + 2 ºC for ten days.

2-Enzyme assay

The tested isolates were propagated firstly on PDA medium at 37 ºC for 3-4 days. Inoculums of fungal isolates were transferred from PDA plates and inoculated on agar medium (gm / L) [(NH4)2 SO4, 0.2; KH2PO4, 0.2; starch, 1.0 and agar agar, 15]. Triplicate cultures were incubated for 72 hours at 37 ºC. Detection the amylase production by using 2% iodine solution was

added to the Petri dishes to detect the clear zones around the colonies against the blue background of substrate. (Pandey,1991; 1992). Select the fungus with the greatest hydrolytic activity for further investigations.

II- Potato Wastes

a- Collection of samples

500 g of each sample of Potato solid wastes were collected from Assuit Manufactory at Assuit governorates. The samples were placed in a double sterile polyethylene bags (to minimizes the loss of water content and provides sufficient aeration),sealed, transferred immediately to the laboratory, kept in cool place (5ºC) until amylase screening .The Chemical analysis and moisture content of samples was directly determined .

b- Chemical Analysis of Potato Wastes

Moisture content, crude fat, ash, crude protein, crude fiber contents were determined according to standard methods A.O.A.C. (1990).

C-Determination of Mineral

1- Digestion of samples

Five grams from each sample was digested by using a mixture of nitric and perchloric acids (Abdel-Akher *et al.*, 1959 and Khan *et al.* 1996).

2- Estimation of Micro and Macro Elements

Micro elements (Fe, Mn, Cu, and Zn) and macro elements (Na, K, Ca, Mg, S and P) were analyzed using atomic absorption spectrophotometer (model GBC 906 AA).

III- Amylase Production by the Best Producer Isolates on Potato Wastes

The most eight highly producer isolates and four isolates isolated from potato wastes were studied (Table III). Screening of fungal species for amylase production on potato wastes (substrate 10 g and 10 ml water for resign moisture content in 250 conical flask, sterilized at 121 ºC for 15 minutes , inoculated by two methods:-

1 ml spores suspension, incubated at 28 ºC for 5 days and examined by addition of 10 ml of sterilized distilled water, mixed and then filtered. The filtrate has used for detection the residue of unused reducing sugar and crude amylase enzyme.

Inoculums in the center of the Petri dish by the tested isolates and the plates were incubated at 28ºC 5 days for determined the production of amylase by clear zone which discussed above (Alva, *et al.*, 2007). The best producer isolate was selected for further investigation.

Determination of reducing sugars

The estimation of starch was carried out using the iodine colorimetric method as described by Tomas and Chamberlain (1980). Reducing sugars were estimated by the dinitrosalicylic acid method using glucose as the standard (Miller, 1959). In the present study, the reducing sugars were described as the sum of the formation due to saccharification and consumption due to fermentation (Abu, *et al.*, 2005).

IV- Optimization And Maximization of Nutritional And Environmental Conditions Affecting Amylase Production By Aspergillus flavus (443) On Potato Wastes

a- Incubation period

To study the effect of incubation period on enzyme production at 28ºC in 250 conical flask- substrate 10g. The enzyme substrate reaction mixture was incubated for different incubation periods (3,5 and 7 days) and enzyme production was recorded.

b-Amount of Substrate Used

Effect of amount of substrate used on enzyme production was measured at different concentrations of potato wastes in the reaction mixture from 1.0 to 50 gm.

c-pH value : Effect of pHs on amylase production was determined by incubating the reaction mixture at pHs values ranging from 3.0 to 11 by using Na OH –HCl one normal.

d-Temperature : Optimum temperature for amylase production was determined by conducting the assay at different temperatures ranging from 10 to 60 ºC.

e-Nitrogen source: Stimulation effect of five different nitrogen sources (1.0 % from each of peptone, urea, ammonium phosphate, ammonium sulphate and casein) on 10 gm potato wastes on amylase production was studied.

f-Carbon source : Stimulation effect of seven different carbon sources (1.0 % from each of lactose, fructose, glucose, maltose, manitol, sucrose and starch) on 10 gm potato wastes on enzyme production was studied.

RESULTS AND DISCUSSION

One hundred and forty-four isolates of filamentous fungi belonging to twenty-two genera and fifty-five species in addition to two species varieties were tested for production of amylase on synthetic medium. Fifty-two isolates were recorded as amylase enzyme producer (Table I) and classified into 3 categories. Three species from *Aspergilli* only have highly productivity and these were *Aspergillus flavus* 443, *A. flavus* var. columnaris961 and *A.oryzae42* (Table I) . In previous studies *Aspergillus flavus* and *A.oryzae* are well known as amylase producers on different substrate as recorded by several workers [Yabuki *et al.*, 1977; Erratt *et al.*, 1984; Arnesen *et al.*, 1998; Viswanathan and Surlikar, 2001; Francis *et al.*, 2003; Nirmala & Muralikrishna, 2003 ; Ramachandran *et al.*, 2004; Kammoun *et al.*, 2008 and Djekrif-Dakhmouche *et al.*, 2006] .

Twenty-four isolates representing 25 isolates have moderate productivity (Table I) *Aspergillus* seven isolates , *Penicilli* , six isolates eleven isolates, two isolates from *Acremonium strictum*; *Alternaria alternata* and others (Table I) .

Twenty-four isolates representing fifteen species and one variety have low productivity of which *Aspergillus* fumigatus var. albus, *A. flavipes*, *A. sydowii* and *A. versicolor*; *Penicillium funiculosum P. purpurogenum* and others (Table 1). It is worthy to mention that some isolates of the same

species variable degrees of amylase production and this depend on the individual isolates.

Table (I): Screening the 144 isolates of filamentous fungi for amylase production on Synthetic Medium

Genus ; species and species variety	***No. of isolates tested***	***+ve isolates***			***Total +ve isolates***	***-ve isolates***
		Low	***Modrate***	***High***		
Absidia *corymbifera*	**1**	**-**	**-**	**-**	**-**	**1**
Acremonium*. strictum*	**3**	**1**	**2**	**-**	**3**	**-**
Alternaria *alternata*	**3**	**-**	**1**	**-**	**1**	**2**
Aspergillus	**36**	**6**	**6**	**3**	**15**	**21**
A. alutaceus	2	-	1	-	1	1
A. candidus	2	-	1	-	1	1
A. Carneus	2	-	1	-	1	1
A. clavatus	1	-	-	-	-	1
A. flavus	2	-	-	1	1	1
A. flavus var. *columnaris*	2	-	-	1	1	1
A. fumigatus	3	-	-	-	-	3
A. fumigatus var. *albus*	4	2	-	-	2	2
A. niger	4	-	-	-	-	4
A. oryzae	1	-	-	1	1	-
A. sydowii	3	3	-	-	3	-
A. tamarii	2	-	-	-	-	2
A. terreus	1	-	-	-	-	1
A. terricola	1	-	1	-	1	-
A. ustus	3	-	2	-	2	1
A. versicolor	3	1	-	-	1	2
Cladosporium	**6**	**-**	**1**	**-**	**1**	**5**
Cl. cladosporioides	3	-	-	-	-	3
Cl. herbarum	3	-	1	-	1	2
Cochliobolus	**6**	**2**	**2**	**-**	**4**	**2**
C. lunatus	3	-	1	-	1	2
C. spicifer	3	2	1	-	3	-
Emerciella	**2**	**-**	**-**	**-**	**-**	**2**
E. nidulans	1	-	-	-	-	1
E. stellata	1	-	-	-	-	1
Fennelia	**3**	**2**	**1**	**-**	**3**	**-**
F. flavipes	1	1	-	-	1	-
F. nivea	2	1	1	-	2	-
Fusarium	**17**	**2**	**1**	**-**	**3**	**14**
F . incarnatum	**1**	**-**	**-**	**-**	**-**	**1**
F. moniliforme	**3**	**1**	**-**	**-**	**1**	**2**
F. oxysporum	**3**	**-**	**1**	**-**	**1**	**2**
F. proliferate	**1**	**1**	**-**	**-**	**1**	**-**
F. sambucusum	**3**	**-**	**-**	**-**	**-**	**3**
F. semitectum	**3**	**-**	**-**	**-**	**-**	**3**
F. solani	**3**	**-**	**-**	**-**	**-**	**3**
Gliocladium	**5**	**-**	**2**	**-**	**2**	**3**
G. . catenulatue	**1**	**-**	**1**	**-**	**1**	**-**
G. roseum	**4**	**-**	**1**	**-**	**1**	**3**

Table (I): Continue

Genus ; species and species variety	No. of isolates tested	+ve isolates			Total +ve isolates	-ve isolates
		Low	Modrate	High		
Macrophomina *phaseolina*	**3**	**-**	**1**	**-**	**1**	**2**
Mucor *circinelloides*	**3**	**-**	**-**	**-**	**-**	**3**
Myrothecium *verrucaria*	**1**	**-**	**-**	**-**	**-**	**1**
Neosartorya fisherii	**4**	**-**	**-**	**-**	**-**	**4**
Paecilomyces *variotii*	**3**	**1**	**-**	**-**	**1**	**2**
Penicillium	**32**	**8**	**7**	**-**	**15**	**17**
P.aurantiogriseum	3	-	1	-	1	2
P. brevicompactum	3	-	-	-	-	3
P. chrysogenum	5	1	1	-	2	3
P. citrinum	4	1	2	-	3	1
P. cyclopium	3	-	1	-	1	2
P. funiculosum	4	4	-	-	4	-
P. italicum	2	-	-	-	-	2
P. janthinellum	3	1	1	-	2	1
P. oxalicum	2	-	1	-	1	1
P. purpurogenum	3	1	-	-	1	2
Scopulariopsis *brevicaulis*	**4**	**2**	**-**	**-**	**2**	**2**
Stachybotrys *chartarum*	**2**	**-**	**-**	**-**	**-**	**2**
Syncephalastrum *racemosum*	**2**	**-**	**-**	**-**	**-**	**2**
Trichoderma. *harzianum*	**3**	**-**	**-**	**-**	**-**	**3**
Trichothecium *roseum*	**3**	**-**	**-**	**-**	**-**	**3**
Trichurus *spiralis*	**2**	**-**	**1**	**-**	**1**	**1**
Total isolates	**144**	**24**	**25**	**3**	**52**	**92**
Number of species	**55 + 2 variety**					
Number of genus	**22**					

H = Isolates which have highly productivity (from 41 to 60 mm clear zone diameter) .
M = Isolates which have moderate productivity (from 21 to 40 mm clear zone diameter).
L = Isolates which have low productivity (from 1 to 20 mm clear zone diameter).

Several of the above species were previously recorded as amylase producers, but with different degrees , as reported by numerous researchers (Prescot & Dunn, 1959 ; Ueda,1981; Hayashida & Teramoto , 1986 ; Bunni *et al.*, 1989; Jensen & Olsen, 1992; Sudo *et al.*, 1995; Okolo *et al.*, 1995; Carlsen *et al*., 1996 a to c; Kaneko *et al.*, 1996 ; Chadha *et al.*, 1997; Arnesen *et al.*, 1998; Goto *et al.*, 1998 ; Nguyen *et al.*, 2000 ; Pederson & Nielson, 2000; Vishwanathan & Surlikar, 2001 ; Aquino *et al.*, 2003; Francis *et al.*, 2003; Moreira *et al.*, 2004 ; Kunamneni *et al.*, 2005 ; Patel *et al.*, 2005; Rahardjo *et al.*, 2005 ; Samborska *et al.*, 2005; Schwab , 2007; Afifi *et al.*, 2008 and Rojas et al ., 2008 and several others) .

Potato wastes

Chemical analysis of potato solid wastes an abundant material that mainly contains (% dm) : were as flows moisture content (77.0)%, crude protein (2.52)%, crude fat (0.13)%, Crude fiber (3.50), Ash (5.31)%, carbohydrate (88. 54) %. The micro and macro element in potato were widely varied (Fe, 87.75; Mn, 5.25; Cu, 11.45 ; Zn, 15.9 ; Na 1350 ; K, 11002; Ca, 2800; Mg, 1560; S ,2295 ;P, 2050 mg/ kg-1 dry mater) Table (II). These results almost in agreement with the results obtained by (Omemu, *et al.*, 2005 and Delgado *et al*,. 2009).

Table (II): Chemical Analysis of the Potato Solid Wastes.

% of the Mean values of chemical composition of the potato wastes						
Moisture content (%)	**77.0**					
Crude protein (Nx6.25) (% dm)	**2.52**					
Crude fat (% dm)	**0.13**					
Crude fiber (% dm)	**3.50**					
Carbohydrate (% dm)	**88.54**					
Ash (% dm)	**5.31**					
Determination of micro and macro element in potato (mg.\ kg-1 Dry Mater)						
Micro elements	**Fe**	**Mn**	**Cu**	**Zn**		
	87.75	**5.25**	**11.45**	**15.9**		
Macro elements	**Na**	**K**	**Ca**	**Mg**	**S**	**P**
	1350	**11002**	**2800**	**1560**	**2295**	**2050**

The screening results of twelve isolates [the most highly amylase producer isolates (3 and 5 of high and moderate productivity, respectively) on synthetic medium and four isolates isolated from potato wastes) for determined their ability to produced amylase enzyme on potato wastes as agro-industrial wastes are shown in Table (III). Of the recorded result, Aspergillus flavus443 was the best amylase producer on the synthetic medium and potato wastes medium. These isolate was selected for further study to optimization and maximization (nutritional and environmental factors) for amylase production.

Table (III): Screening of Twelve Isolates for Amylase Production on the Potato Wastes:-

Serial no.		Tested fungal genus & species With No. of strain (AUCC)	Reducing sugars (g/L)	Mean diameters of clearing zone (mm)
The Best amylase Producer Isolates on Synthetic Medium	1	***Aspergillus flavus*$_{443}$**	**0.298**	**24.5**
	2	***A. flavus var columinaris*** $_{461}$	**0.456**	**24.1**
	3	***A. oryza*** $_{42}$	**0.702**	**20.5**
	4	***A. ustus*$_{1450}$**	**0.730**	**19.5**
	5	***Cladosporium herbarum*$_{3413}$**	**0.650**	**22.0**
	6	***Cochliobolus spicifer*$_{143}$**	**1.140**	**14.0**
	7	***Gliochlodium catenulate*$_{257}$**	**1.154**	**13.5**
	8	***Penicillium cyclopium*$_{723}$**	**0.678**	**21.2**
Isolates Isolated From Potato Wastes	9	***Aspergfillus flavus*$_{MAF}$**	**0.762**	**18.5**
	10	***A. niger***	**0.762**	**18.5**
	11	***Fusarium moniliforme*** $_{S}$	**1.154**	**13.5**
	12	***Fusarium*** $_{L}$	**1.154**	**13.5**
Control			**0.460**	**23.6**

Table (IV) shows that the best environmental factors for amylase production by *Aspergillus flavus*443 on potato wastes medium were: 3 days incubation period , pH 5 and 40 ºC incubation temperature . Our results are almost in agreement with previous studies on amylase production by some species of filamentous fungi by (Prescot & Dunn, 1959; Yamasaki *et al.*, 1977; Tomas & Chamberlain,1980; Bergmann *et al.*,1988; Hayoshida *et al.*, 1988 ;Okolo *et al.*,1995 ;Abu *et al.*, 2005 ; Haq *et al.*, 2006 ;Alva,*et al.* ,2007; Mona Gouda & Elbahloul ,2008 ; Rojas *et al*.,2008 and Delgado *et al.*,2009).

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Also Stimulation of amylase production was obtained when manitol (1.0 %) or sucrose (1.0 %) were used as a carbon source (from1.2 to 25.5 or 28 mm clear zone and 1.2 to 0.2 gm/L reducing sugars) and casein followed by ammonium sulphate, urea, ammonium phosphate and peptone as nitrogen sources (21, 75- 25.5 mm clear zone; and 0.65 – 0.27 gm/L reducing sugars) as nitrogen sources.

The best amount of potato waste was used for amylase production was 15 – 50 gm waste in 250 ml conical flask pluss 10 ml sterilized water (27.3 – 28.0 mm clear zone , 0,23 – 0.20 gm/L reducing sugars).

Several workers studied the effect of some carbon and nitrogen sources for amylases production by several fungi such as some members of *Aspergillus*, Penicillium and Trichoderma (Kammoun *et al.*, 2008 and Mona Gouda & Elbahloul ,2008).

Haq *et al.*, (2006) found that Trichoderma viride was further optimized for enhanced production and increased in amylase production was observed when sweet potato starch was used as carbon source. No enhancement in production was taken place by replacing ammonium sulphate with any other nitrogen source. USDA (2008) and Delgado *et al.*(2009) observed that potato starch is very suitable substrate for amylase production by several fungi .

In conclusions, potato waste (as agro-industrial waste) is economically important and can be used as a substrate for amylase production by several fungi and enzyme was used in numerous industries such as food and health care, chemical industry, polymer synthesis, pharmaceutical industry and the energy sector.

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إنتاج إنزيم الاميليز بواسطة الفطريات الخيطية

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لمعرفة قدرة بعض الفطريات الخيطية على إنتاج إنزيم الأميلز تم استخدام ١٤٤ عزلة منها فتبين قدرة ٥٢ عزلة فقط من بين العزلات المختبرة على إنتاج الإنزيم ولكن بدرجات مختلفة وذلك باستخدام طريقة الأطباق الصلبة , ثم تم استخدم ١٢ عزلة من الفطريات السابقة التي ثبت قدرتها على إنتاج إنزيم الاميليز (٨ عزلات عالية الإنتاج , ٤ عزلات من مخلفات البطاطس) على مخلفات البطاطس لاختبارها لإنتاج إنزيم الاميلز . وسجلت الدراسة قدرة فطر *Aspergillus flavous*(443) كأفضل منتج لإنزيم الاميلز على بيئة الوسط الغذائي وعلى مخلفات البطاطس .وقد تم دراسة الظروف البيئية و التغذاوية للفطر والمؤثرة على إنتاج الانزيم فكانت افضل الظروف كالتالى (٥٠ جم مخلفات بطاطس , ١٠ مل ماء مقطر , سكر مانيتول ١ % كمصدر كربونى , الكازين ١% كمصدر نتروجين , وعند آس هيدروجيني ٥,ويتم التحضين على٤٠ ° م لمدة ثلاث أيام) .

Table (IV): Optimization and Maximization of Both Nutrition and Environmental Factors Affecting Amylase Enzymes Production by *Aspergillus flavus* (443) as highly Producer Strain Tested On Potato Wastes.

Environmental factors									**Nutritional factors**								
Incubation period			**pH values**			**Incubation temperature**			**Nitrogen sources**			**Carbon source**			**Amount of substrate used**		
Days	***R. s.**	****M. d. c .z**	**pH**	***R.S.**	****M. d. c .z**	**◦C**	***R.S.**	****M. d. c .z**	**Name**	***R. s.**	****M. d. c .z**	**Name**	***R.s.**	****M. d. c .z**	**gm**	***R.s.**	****M. d. c .z**
3	**0.234**	**27**	3	0.722	19.8	10	0.754	18.75	Peptone	0.650	22.1	Lactoe	1.056	16.1	1	0.722	20.0
5	0.276	25.5	4	0.714	20.0	20	0.630	22.5	Urea	0.440	24.5	Fructose	1.200	12.0	2.5	0.650	22.0
7	0.298	25.0	**5**	**0.448**	**24.5**	30	0.480	23.12	Ammonium phosphate	0.658	21.75	Glucose	1.216	11.5	5	0.640	22.3
			6	0.710	20.3	**40**	**0.294**	**25.12**	Amonium sulphate	0.432	24.7	Maltose	1.088	15.3	15	0.228	27.3
			7	0.714	20.2	50	1.040	16.5	**Casine**	**0.276**	**25.5**	**Manitol**	**0.202**	**28.0**	25	0.218	27.5
			8	0.718	20.1	60	1.060	16.0				Sucrose	0.702	25.5	**50**	**0.202**	**28.0**
			9	0.722	20.0							Starch	1.200	12.0			
			10	0.686	21.0												
			11	1.060	16.0												

***R.s. = Residue of the reducing sugars in the culture medium (g/l)**

****M. d. c.z = Mean diameters of clearing zone(mm)**