ORIGINAL RESEARCH PAPER | Microbiology | Volume : 6 | Issue : 10 | October 2016 | ISSN - 2249-555X | IF : 3.919 | IC Value : 74.50

Enzymatic Study of *Fusarium Moniliforme*, Sheldon Var. Subglutinans, Isolated from Wilted Sugarcane Plant

KEYWORDS: Fusarium moniliforme, enzyme, amylase, cellulase, lipase

Rakeshkumar R. Panchal

Department of Microbiology, M. B. Patel Science College, Anand-388001, Gujarat, India.

ABSTRACT *Fusarium moniliforme Sheldon, a wilt pathogen of sugarcane was tested for its amylase, cellulase and lipase activity. Enzyme production from Fusarium moniliforme Sheldon was carried out in 50 ml mineral medium containing g/l … in 250 ml Erlenmeyer flask using submerged production in shake flask culture. Prepared media flasks were inoculated by young spore suspension to get 106 no. of spores/ml of medium and incubated at 25 °C, 120 rpm for 7 days. The highest amylase activity of 12 U/ml was observed on seventh day. The cellulose activity was determined as carboxy methyl cellulase (CMCase), filter paperase (FPase), exoglucanase and β-glucicidase. The fungus produced the maximum 0.16 U/ml of CMCase after 72 h. FPase, exoglucanase and β-glucicidase was recorded 0.253 U/ml, 0.042 U/ml and 0.586 U/ml after 7 days incubation, respectively. The lipase production of 11 U/ml was observed on 7th day from Fusarium moniliforme Sheldon*

Introduction

The genus *Fusarium* produces wilt diseases and root rot in a wide variety of plants and the ability of the pathogen to cause wilt symptoms is correlated to its enzyme production (Cooper 1984). Usually these fungi preferentially degrade carbohydrates and pectin in wood; different enzymes involved in cellulose, hemicellulose and pectin degradation have been characterized previously (Alconada&MartõÂnez 1994). Several plant pathogenic fungi, including *Fusarium*, produced cell wall degrading enzymes in the host tissue (Garcia-Maceira et al. 2001)

Enzymatic hydrolysis is an attractive approach for the utilization of cellulosic materials and production of reducing sugars which are then available for use as chemical feedstock, as sweetener in the food industry or as growth substrate for various organisms in the production of solvents, organic acids, antibiotics and single cell protein (Ward and Moo-Young, 1989).

The production of cellulase was reported from a mutant strain (NTG-19) of *Fusarium oxysporum*(Kuhad et al. 1994). Ruka et al. (1998) investigated *Fusarium culmorum* for its ability to produce proteolytic, amylolytic and lipolytic enzymes.The production of extracellular laccase and aryl-alcohol oxidase from *Fusarium proliferatum* strain was reported (Regalado et al. 1999).

Materials and methods

Amylase production

The production of amylase was carried out in submerged condition. The 100 ml production medium containing (g/L): KH_2P0_4, 2.0; $CaCl_2$, 0.3; $MgS0_4.7H_2O$, 0.003; $FeS0_4.7H_2O$, 0.005; $MnS0_4.H_2O$, 0.016; $ZnS0_4.7H_2O$, 0.014; $CoCl_2$, 2.0; 1.0. supplemented with 1 % (w/v)starch as the carbon source in 250 ml Erlenmeyer flasks were sterilized at 121 °C, 15 lbs for 20 min. The media flasks were inoculated with fresh spore suspension of *Fusarium moniliforme* Sheldon to get 10^6 spores/ml of production medium. The inoculated flasks were incubated at 25 °C, 120 rpm on a rotary shaker for 7 days. 2 ml sample was withdraw after every 24 h and fungal mycelia were removed by filtration. The filtrate was analyzed for amylase production.

Amylase assay

The determination of amylase was carried out on the basis of starch hydrolyzed and reducing sugar produced by the enzyme present in the filtrate. Amount of sugar released was estimated by DNSA reagent (Miller, 1959). Appropriately diluted 1.0 ml of filtrate was added to 1.0 ml of 0.5 % (w/v) starch solution in phosphate buffer (50 mM, pH-6.0). In control tube 1.0 ml buffer was added instead of enzyme. Both tubes were incubated at 40 °Cin waterbath for 10 min. After incubation, 2.0 ml ofDNS reagent was added to stop the reaction. The reducing sugar produced during the incubation was estimated as per DNSA method. And the amount of enzyme produced was calculated using the standard glucose (0-1.0 mg/ml) calibration curve prepared by DNSA method.

One unit enzyme was defined as the amount of amylase that releases 1.0 µmole of reducingsugar or D-glucose per minute under the assay conditions.

Cellulase production

The cellulase production was done as described by Sternberg(1976). The 100 ml production medium containing (g/L): $(NH4)_2S0_4$, 1.4; KH_2P0_4, 2.0; Urea, 0.3; $CaCl_2$, 0.3; $MgS0_4.7H_2O$, 0.003; $FeS0_4.7H_2O$, 0.005; $MnS0_4.H_2O$, 0.016; $ZnS0_4.7H_2O$, 0.014; $CoCl_2$, 2.0; Cellulose, 20.0,Proteose peptone, 1.0. All the ingredientsexcept urea were dissolved and the pH of the medium was adjusted to 5.2 and it was sterilized at 121 °C, 15 lbs for 20 minutes. The media flasks were inoculated with fresh spore suspension of *Fusarium moniliforme* Sheldon to get 10^6 spores/ml of production medium. The inoculated flasks were incubated at 25 °C, 120 rpm on a rotary shaker for 7 days. 2 ml sample was withdraw after every 24 h and fungal mycelia were removed by filtration. The filtrate was analyzed for cellulase production.

CMCase assay

To 1.0 ml of 1.0 % (w/v) carboxy methyl cellulose in acetate buffer (50 mM, pH-5.0), 1.0 ml of filtrate was added. In control tube 1.0 ml buffer was added instead of enzyme. Both tubes were incubated at 50 °C for 30 min. After incubation, 2.0 ml of DNS reagent was added to stop the reaction. The reducing sugar was estimated by DNSA method and calculated using the standard glucose (0-1.0 mg/ml) calibration curve.

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One unit of enzyme is the amount of CMCase produced 1 µmole of glucose per minute under the assay conditions.

FILTER PAPERASE (FPase) assay

Filter paperase (FPase) was measured by taking 50 mg of Whatmanno-1 filter paper (rolled 1x6cm strips) in 1.0 ml of citrate buffer (50 mM, pH-4.8). To this 1.0 ml ofculture filtrate was added and incubated at 50 °C for 1 hour and the liberated reducing sugar was measured by DNSA method using standardglucose (0-1.0 mg/ml) calibration curve.

One unit of enzyme is the amount of FPase produced 1 µmole of glucose per minute under the assay conditions.

Cotton hydrolysis

For cotton hydrolysis assessment, 50 mg absorbent cotton was taken in 1.0 ml of citrate buffer (50 mM, pH-4.8). To this 1.0 ml of culture filtrate was added and incubated at 50 °C for 1 hour and the liberated reducing sugar was measured by DNSA method using standard glucose (0-1.0 mg/ml) calibration curve.

One unit of exo-glucanase is the amount of enzyme released mg of glucose per hour under the assay conditions.

β-glucocidase assay

β-glucosidaseassay was carried out as described by Hosel et al. (1978), using p-nitrophenyl-β-D-glucoside (PNPG) as substrate. To 0.9 ml of 2.0 mMPNPG in citrate buffer (50 mM, pH-4.8), 0.1 ml of diluted filtrate was added and incubated at 50°C for 20 minutes. After incubation, 2.0 ml of 1 M Na_2C0_3 was added absorbance was measured at 420nm.

One unit of β-glucosidase is defined as the amount of enzyme liberating 1 µmole of p-nitrophenolper min under assay conditions.

Lipase production

The production of lipase was done as described by Peter Rapp (1995).The 100 ml production medium containing (g/L): $MgS0_4$. $7H_2O$, 0.5; KH_2P0_4, 1.0; $NaN0_3$, 3.0; yeast extract, 1.0; Peptone, 30; Glucose, 10; Tributyrin, 10; pH of the medium was adjusted to 5.2 and it was sterilized at 121 °C, 15 lbs for 20 minutes. The media flasks were inoculated with fresh spore suspension to get 10^6 spores/ml of production medium. The inoculated flasks were incubated at 25 °C, 120 rpm on a rotary shaker for 7 days. 2 ml sample was withdraw after every 24 h and fungal mycelia were removed by filtration. The filtrate was analyzed for lipase production.

Lipase assay

Lipase assay was done as described by Shah and Desai (2002). Substrate emulsion was prepared by taking 75ml of 2% polyvinyl alcohol and 25 ml olive oil in 250ml flask. This mixture was homogenized using vortex mixture. To 5.0 ml freshly prepared substrate emulsion, 4.0 ml sodium phosphate buffer (50mM, pH-8.0) was added in 100 ml flask and was preincubated at 32 °C for 10 minutes. 1.0 ml enzyme filtrate was addedto this and incubated at 32°C for 30 min in reciprocating waterbath.The reaction was terminated by adding 20ml of acetone and then titrated against 0.1 N NaOH (0.05 M) usingphenolphthalein as an indicator.

One unit of lipase was defined as the amount of enzyme that release 1µmole of fatty acids per minute from emulsion under assay conditions.

Result and discussion:

Fusarium moniliforme Sheldon, wilt pathogen of sugarcane, showed very low level of amylase activity. Very low activity was seen on third day which was only 4 u/ml and at the end of the seventh day also there was not much increase in the activity and the value was 12 u/ml as shown in table-1. Low amylase activity in *Fusarium moniliforme* is expected as the starch content in the sugarcane is less. Amylase produced by *F.culmorum*when grown on soybean was 0.5 u/ml and the same amount produced when grown on starch (Rucka, M.*et.al.*(1998).

Table 1: Amylase activity of *Fusarium moniliforme*.

Days	pH	Mycelial dry weight mg/ml	Amylase activity unit/ml	Specific amylase activity unit/mg
1	5.60	2.0	00	0.000
2	5.20	3.1	00	0.000
3	5.22	4.0	04	1.000
4	5.68	5.2	07	1.346
5	5.95	6.5	08	1.231
6	6.01	7.9	11	1.392
7	6.08	9.1	12	1.319

As shown in table -2 the cellulose activity observed was also low. The cellulose activity was measured in terms of endoglucanase, exoglucanase, β – glucosidase and total cellulase activity. The activity of all the components increased from the day one up to 7th day. Among the components, β – glucosidase activity was more. Cellulase activity in fungal plant pathogen, when studied in liquid medium, in batch process was low (Kumar and Lonsane, 1988).Kuhad, Manchanda and Singh (1999) mutated *Fusarium oxysporum* and could produce 15.1 u/ml CMCase and 9.0 u/ml FPU.

Table 2: Cellulase activity of *Fusarium moniliforme*.

Days	pH	FPase u/ml	CM-Case u/ml	Exoglucanase u/ml	B – glucosidase u/ml
1	5.48	0.020	0.04	0.017	0.235
2	5.29	0.091	0.104	0.0188	0.292
3	5.40	0.106	0.160	0.0210	0.342
4	5.77	0.130	0.155	0.0265	0.396
5	5.80	0.172	0.131	0.0351	0.415
6	5.98	0.197	0.134	0.0374	0.503
7	6.10	0.253	0.110	0.0402	0.586

No lipase activity was observed on the first day, although growth was initiated. From second day onwards the lipase activity increases up to 6th day. The activity was growth associated and maximum activity and growth were recorded on the sixth day. On the seventh day the lipase activity decreases, but not the growth. The decrease in lipase activity may be due to the end product inhibition. Very little lipase activity was present on the seventh day in the control, having glucose as the carbon source. The growth of mycelia was abundant in the control.

Table 3: Lipase activity of *Fusarium moniliforme*.

Days	pH	Mycelial dry weight mg/ml	Lipase activity u/ml	Specific lipase activity u/mg
1	5.5	1.9	0.0	00
2	5.9	4.3	0.8	0.186
3	5.7	6.5	2.6	0.400
4	5.8	7.7	6.2	0.805
5	6.3	8.3	10.3	1.241
6	6.3	8.6	12.1	1.407
7	6.2	8.7	11.2	1.287

Volume : 6 | Issue : 10 | October 2016 | ISSN - 2249-555X | IF : 3.919 | IC Value : 74.50

The lipase production and the growth pattern of *F. moniliforme* Sheldon matched with that of *F. oxysporum* (Rapp,1995). Studies carried out in shake flask with *F. oxysporum* , showed little constitutive lipase activity associated with the mycelium but higher inducible extra cellular activity which was growth associated. The amount of lipase activity observed in *F. oxysporum* was very high as compare to that in *F.moniliforme* in our studies. End product inhibition and repression of lipase was observed in F. oxysporum. No lipase activities was detected when F. culmorum grown on soybean as well as starch as 'C' source (Rucka, M.*et.al.*1998)

The production of polygalacturonase, pectatelyase, carboxymethylcellulase, xylanase and amylase from *Fusarium moniliforme* NCIM1276 was increased by 3, 2, 11, 10 and 4-fold respectively on semi-solid medium containing wheat bran and orange pulp (Niture and Pant, 2007).

SUMMERY:

The present investigation is concluded with finding of facts of production of hydrolytic enzymes by sugarcane wilt pathogen *Fusarium moniliforme*. The highest amylase activity of 12 U/ml was observed on seventh day. The fungus produced the maximum 0.16 U/ml of CMCase after 72 h. FPase, exoglucanase and β-glucicidase was recorded 0.253 U/ml, 0.042 U/ml and 0.586 U/ml after 7 days incubation, respectively. The lipase production of 11 U/ml was observed on 7th day from *Fusarium moniliforme* Sheldon. These enzymes are the important means of the mechanism of pathogenesis of this fungi.

Acknowledgement:

We are thankful to Department of Biosciences , Veer Narmad South Gujarat University, Surat and M.B.Patel Science College , Anand for providing all the facilities.

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