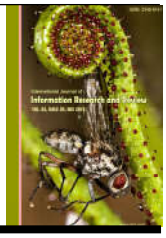
IJIRR

International Journal of Information Research and Review
Vol. 03, Issue, 05, pp. 2330-2333, May, 2016



Research Article

ISOLATION AND SCREENING OF CELLULOLYTIC ACTIVITY FROM PLASTIC DUMPED SOILS

***Sumathi, T., Akula Srilakshmi and Sai Gopal, D.V.R.**

Department of Virology, Tirupati, Sri Venkateswara University, Tirupathi 517502. India

ARTICLE INFO

Article History:

Received 03rd February 2016
Received in revised form
22nd March 2016
Accepted 29th April 2016
Published online 30th May 2016

Keywords:

Industrial Enzymes, cellulases, FPAase, CMCase, β Glucosidase, Cochliobolus sps.

ABSTRACT

Microorganisms, especially fungi are the leading producers of an array of industrial enzymes. Microbial cellulases are the enzymes that are sold in large volumes due to their enormous applications in biochemical industry. Selection of the right organism plays a predominant role in high yield of desirable enzymes. Hence an attempt was made in the current study to isolate and screen novel fungi from plastic dumped soil for the optimal production of cellulases. In this study eight fungal cultures were screened, of these three cultures were found to exhibit cellulolytic activity. The most potent isolate which exhibited maximum cellulolytic activity was screened. The activities of cellulases were determined by Filter paper (FPA), Carboxy-methyl cellulase (CMCase) and β-D-glucosidase assays. The total extracellular protein contents of the fungal filtrates and biomass of the fungal cultures were also determined. A significant cellulase production, extracellular protein, fungal biomass was noticed with *Cochliobolus sps* organism compared to the remaining fungal cultures.

INTRODUCTION

Plastics are defined as the polymers (solid materials) which on heating become mobile and can be cast into moulds. They are non-metallic moldable compounds and the materials that are made from them can be pushed into any desired shape and sizes (Seymour, 1989). Commonly plastics are used in many purposes including packaging, disposable diaper backing, agricultural films and fishing nets. Plastics and their use has become a part in all sectors of economy (Sabir, 2009) Infrastructure such as agriculture, telecommunication, building and construction, consumer goods, packaging, health and medical are all high growth areas that ensures present demand for plastics. Plastic is the mother industry to hundreds of components and products that are manufactured and used in our daily life like automobiles parts, electrical goods, plastic furniture, defense materials, agriculture pipes, packages and sanitary wares, pipes and fittings, tiles and flooring, artificial leathers, bottles and jars, PVC shoes and sleepers hundreds of household items. Plastics are resistant to microbial attack, because their short time of presence in nature evolution could not design new enzyme structures capable of degrading synthetic polymers (Mueller, 2006). Nowadays, a wide variety of petroleum-based synthetic polymers are produced worldwide to the extent of approximately 140 million tons per year and remarkable amounts of these polymers are introduced in the ecosystem as industrial waste products (Shimao, 2001).

Corresponding author: D.V.R. Sai Gopal et al.,
Department of Virology, S.V.University, Tirupati.

This growing concern about degradable polymers has raised and promoted research activity world-wide to either modify current products to promote degradability or to develop new alternatives that are degradable by any or all of the following mechanisms: biodegradation, photo degradation, thermal degradation and environmental erosion (Kawai, 1995). In 1980′s, scientists started to look if plastics could be designed to become susceptible against microbial attack, making them allowed to degrade in a microbial active environment. Biodegradable plastics has opened the way for new considerations of waste management strategies since these materials are designed to degrade under environmental conditions or in municipal and industrial biological waste treatment facilities (Augusta *et al.,* 1992 and Witt *et al.,* 1997). In these present studies the plastic dumped soil was collected fungal organisms and the activity of cellulolytic enzyme produced in different incubation days and their related enzymes are isolated and screening was identified.

MATERIALS AND METHODS

In these present studies six fungal cultures were isolated from plastic dumped soil (collected from renigunta, Tirupati, Andhra Pradesh. India.) by serial dilution method. 1 g of soil was transferred to 10 ml of distilled water in test tubes. Dilutions were made up to $10^{-6}$and 0.1 ml of soil suspension was spread on to the sterilized Czapek-Dox agar medium with following composition (g/l): sucrose – 30, $NaNO_3$ – 2, K_2HPO_4 - 1, $MgSO_4$ – 0.05, KCl – 0.5, $FeSO_4$– 0.01, Agar agar - 20.After autoclaving at 121 °C and 15 lbs. pressure, 20 ml of sterile

medium were transferred to sterile Petri plates and allowed for solidification. After solidification of the medium 0.1 ml of soil suspension was spread with the help of spreader and incubated at 28 °C for 7 days. The fungal cultures grown on the medium were transferred on to the Potato dextrose agar slants and maintained at 4 °C for further studies.

Screening of Cellulolytic fungi: The isolated fungal cultures were screened for their ability to produce cellulases complex following the method of Teather & Wood (1982). Czapek-Dox medium used in this method contained (g/l): sucrose – 30, $NaNO_3$ – 2, K_2HPO_4 - 1, $MgSO_4$ – 0.05, KCL – 0.5, $FeSO_4$ – 0.01, Carboxy- methyl cellulose – 1%, Agar agar - 20. After autoclaving at 121°C and 15 lbs. pressure, the medium was poured into Petri plates and allowed to solidify. Cavities of 6 mm size were made in the solidified medium and inoculated with 0.1 ml of spore suspension prepared from 7 day old slants. The plates were incubated at room temperature (28 ± 2 °C) for three to seven days allow fungal growth and then again incubated at 50°C for 18 hours. After incubation, the plates were flooded with 1% Congo red solution and shaken at 50 rev/min for 15 min. The Congo red solution was then poured off; plates were flooded with 1 N NaOH and shaken again at 50 rev/min for 15 minutes. Finally 1 N NaOH was also poured off and plates were observed for the formation of yellow colored zones around the inoculated wells.

Identification of fungal culture: Total six fungal cultures appeared in plates was further purified by sub culturing number of times on potato dextrose agar plates and finally maintained on the same slants. Cultural characteristics such as color and size of colonies of fungal cultures during the growth were monitored and recorded (Domsch *et al.,* 1980).

Production of cellulase enzyme by isolated fungal culture: Among three fungal isolates only one fungal was used to screen the cellulolytic activity. The screened fungal isolate was used to know their potential for cellulase production on Czapek-Dox broth medium supplemented with 1% (w/v) cellulose. 100 ml of Czapek-Dox was distributed into separate 250ml Erlenmeyer conical flasks. After sterilization, the flasks were inoculated with spore suspensions of four fungal isolates. The flasks were incubated at 32°C on a rotary shaker at 120 rpm for with different days (2^{nd}, 4^{th}, 6^{th}, and 8^{th} day). For all the experiments triplicates were maintained. After incubation, the contents of the flasks were passed through whattman filter paper No.1 to separate mycelial mat and culture filtrate. The filtrate thus obtained was used for the estimation of biomass, extracellular protein content, total soluble sugar and total cellulase activity such as Fpase, endoglucanase and β-glucosidase.

Analytical Methods

Extracellular protein content

After 7 days of incubation the culture filtrates was derived from different experiments. An aliquot of this culture filtrates was used for estimation of extra cellular protein content according to the method of Lowry *et al.,* (1951). Bovine serum albumin was used as protein standard. Suitable aliquots of filtrate were mixed with 5 ml of alkaline solution. After 30 min, 0.5 ml of appropriately diluted folin-ciocalceteal reagent was added. The color developed was read at 640 nm using visible spectrophotometer.

Estimation of total soluble sugar

The total soluble sugar content in the culture filtrates was carried out according to the method of Miller (1959). Glucose was used as sugar standard. Suitable aliquots of culture filtrates were mixed with 3 ml of DNS reagent. The contents were boiled vigorously in a boiling water bath for exactly five minutes and the color developed was read at 540nm using spectrophotometer.

Determination of fungal biomass

After appropriate incubation period (7 days) the contents of the flasks was aseptically passed through pre-weighed Whattmann No.1 filter paper to separate mycelial mat and culture filtrate. The filter paper along with mycelial mat was dried at 70°C in an oven until constant weight and the weight was recorded. Difference between the weight of the filter paper bearing mycelial mat and weight of pre-weighed filter paper represented fungal biomass, which was expressed in terms of dry weight of mycelial mat (mg/flask).

Cellulase enzyme assay

The filtrate obtained after removal of mycelial mat by filtration through Whattman Filter paper No.1 was used as an enzyme source. Flasks with the growing culture of fungal isolates were withdrawn at every 7-day interval for processing.

Filter paper assay (FPA)

Filter paper activity of the culture filtrates was determined according to the method of Mandels and Weber (1969). Whattmann filter paper strips containing 50mg weight was suspended in one ml of 0.05 M sodium citrate buffer (pH 4.8) at 50°C in a water bath. Suitable aliquots of enzyme source was added to the above mixture and incubated for 60 minutes at 50°C.

After incubation, the liberated reducing sugars were estimated by the addition of 3, 5-Dinitrosalicylic acid (DNS). After cooling, color developed in tubes was read at 540 nm in a spectrophotometer. Appropriate control without of enzyme was simultaneously run. Activity of cellulase was expressed in filter paper units. One unit of filter paper unit (FPU) was defined as the amount of enzyme releasing one μ mole of reducing sugar from filter paper /ml /hour.

Endoglucanase assay

Activity of endoglucanase in the culture filtrate was quantified by carboxymethyl cellulase method (Ghosh, 1987). The reaction mixture with 1.0 ml of 1% carboxymethyl cellulose in 0.2 M acetate buffer (pH 5.0) was pre-incubated at 50°C in a water bath for 20 minutes.

An aliquot of 0.5 ml of culture filtrate with appropriate dilution was added to the reaction mixture and incubated at 50°C in water bath for one hour. Appropriate control without of enzyme was simultaneously run.

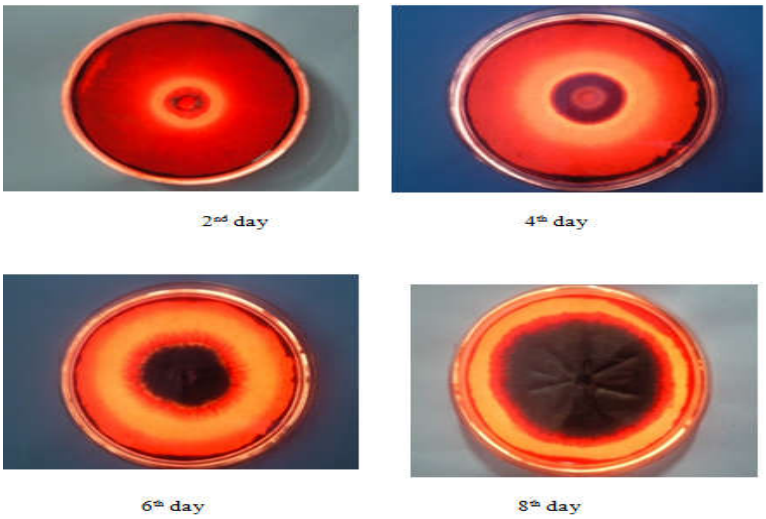


Figure 1. Screening of cellulolytic fungal culture on CMC agar plates (With different incubation days)

Table. 1. Isolation of fungal cultures from plastic dumped soils.

S.NO	Sample	Dilution	Colony size (cm)	Shape	Colour of colony
1.	PS_4	10^{-4}	0.7	Raised circular	Greyish green and white margin
2.	PS_4	10^{-2}	3.5	Circular flat	Cottony appearance and greyish green at Center
3.	PS_4	10^{-2}	1.0	Circular flat	Bluish green

Table 2. Biometric feature cellulolytic isolated fungi-2 on different days

S.NO	Incubation time (in days)	Colony diameter (cm) on CMC agar plate	Zone of hydrolysis on CMC agar plates (cm)
1.	2^{nd} day	1.7	3.0
2.	4^{th} day	4	5.0
3.	6^{th} day	5.5	7.0
4.	8^{th} day	7	9.0

Table 3. Cellulolytic activity of isolate-2 fungi with different day's incubation period

S.NO	Assay of Cellulolytic fungi	2^{nd} day	4^{th} day	6^{th} day	8^{th} day
1.	Filter paper assay (U/ml/hour)	23.88 ± 0.09	52.2 ± 0.05	57.23 ± 0.05	28.99 ± 0.06
2.	Endo gluconase activity	50.76±0.02	89.93±0.03	97.87 ± 0.01	50.45±0.07
3.	Protein (mg/ml)	1040 ± 0.06	1570± 0.03	2720 ± 0.06	1530±0.00
4.	Glucose(µg/ml)	8650 ±0.03	12100 ±0.01	13650 ±0.01	5493.3±0.04
5.	Biomass(g/ml)	1.18 ±0.04	1.42 ±0.02	1.77±0.03	1.55 ±0.02
6.	β- glucosidase (U/ml/hour)	0.02 ±0.01	0.03 ± 0.02	0.08 ± 0.01	0.01 ±0.00
7.	CMCase activity (U/ml/hour)	14.34 ± 0.02	46.66 ± 0.02	81.00±0.05	12.23 ±0.03

Note: Triplicates values mean ± standard deviation.

a) Fpase - One filter paper unit (FPU) was defined as the amount of enzyme releasing one micromole of reducing sugar from filter paper /ml / hour.

b)CMCase -One unit of endoglucanase activity was defined as the amount of enzyme releasing one micromole of reducing sugar from Carboxy methyl cellulose /ml /hour.

c)β- Glucosidase - One unit of β–glucosidase activity was defined as the amount of enzyme liberating one micromole of p – nitro phenol from p-nitro phenyl β - D-gluco pyranoside /ml /hour under.

β-D- Glucosidase assay

Activity of β-glucosidase in the culture filtrate was based on the method of Herr (1979). For the determination of β-D-glucosidase activity the assay mixture contained 0.2 ml of 5mM p-nitro phenyl β-D-gluco pyranoside (PNPG,) in 0.05 M citrate buffer pH 4.8 and 0.2 ml of diluted enzyme solution with appropriate controls. After incubation for 30 min at 50°C, the reaction was stopped by adding 4 ml of 0.05 M NaOH-Glycine buffer (pH 10.6) and the yellow colored p-nitro phenol liberated was determined at 420 nm in spectrophotometer. One unit of β-glucosidase activity was defined as the amount of enzyme liberating one µ mole of þ-nitro phenol /ml /hour under standard assay conditions.

The reducing sugar produced in the reaction mixture was determined by dinitro salicylic acid (DNS) method (Miller, 1959). 3, 5-Dinitro-salicylic acid reagent was added to aliquots of the reaction mixture and the color developed was read at wavelength 540 nm in a spectrophotometer (ELICO, SL 171). One unit of endoglucanase activity was defined as the amount of enzyme releasing one µ mole of reducing sugar /ml /hour.

RESULTS AND DISCUSSION

The fungal cultures isolated from plastic dumped soil were identified (Table 1). Among six cultures only one culture were used for the screening of cellulolytic activity with different interval days. (2^{nd}, 4^{th}, 6^{th}, and 8^{th}day). The fungal culture

examined produced zone of hydrolysis with different incubation periods and the results were represented (Table.2 and figure.1). (Teather and Wood, 1982; Bradner *et al*. 1999; Dale Peciulyte, 2007).The fungal isolate *Cochliobolus sp*. (isolate-2) was tested in this present study high activity against CMC as indicated by zone of hydrolysis which was 9cm on 8th day. On 2nd day, 4th day and 6th day exhibited lower activity against CMC as the diameter of zone of hydrolysis was 3.3, 5.5, and 7.8 cm respectively. When the cellulase activity was tested quantitatively with dinitrosalicyclic acid reagent, the results obtained with Congo red and dinitrosalicyclic acid reagent method. (Lynd *et al.* 2002). Similar reports with other fungal cultures have been already reported by Naveen Kumar *et al*., (2013). , Lekh Ram *et al*., (2014). 1.6 ± 0.6 cm by *Aspergillus niger,*(areca nut husk waste), and 0.7cm of colony diameter by PISS- 3 (paper industry soil sample) respectively. In the current study as maximum zone diameter was expressed by fungal isolate 2.

The isolated fungal culture was identified up to genus level based on their morphological represented in Table.1. The isolated fungal culture was grown on Czapex- Dox broth medium amended with 1% cellulose as substrate (Dale Peciulyte, 2007). For the production of cellulases and the results were shown table.3. In these present study *Cochliobolus sp.* (Isolate 2) (GenBank Accession No:805051) produced high titers values on 6th day of Filter paper assay (57.23 U/ml) Carboxy –methyl cellulase assay (CMCase) (81U/ml) β-glucosidase (0.08U/ml) total soluble sugar (13650µg/ml), biomass (1.77g/ml) and extracellular protein content (2680mg/ml) in comparison with remaining fungal culture incubation days. Fpase activity of 52.2 U/ml and CMCase of 46.66U/ml were exhibited on 4th day, which showed its efficiency. When compared with cellulose activity of 6th day, 2nd and 8th day lower activities of Fpase (23.88, 28.99 U/ml), CMCase (14.34, 12.23U/ml) and β- glucosidase (0.02-0.01) as well as lower contents of soluble sugar (600, 400µg/ml) and whereas least dry mycelium weight of fungal mat (1.18g/ml) was recorded on the 2nd day.

Conclusion

Fungi are considered as the primary decomposers of soils. The use of fungi have a number of advantages than bacteria and yeast, the most important of which being their capability to utilise a wider variety of cellulosic substrates generally regarded as wastes form industries and agriculture fields. Presently our studies investigated the efficacy of fungal cultures isolated from plastic dumped soil for the production of extracellular cellulases. Out of eight fungal isolates only three isolates were showing the positive result for cellulase activity. Cellulase production was carried out on Czapex – Dox medium for varying time intervals. The fungal culture exhibiting highest cellulolytic activity was identified as *Cochliobolus sps*. To our knowledge this may be the first report on cellulase production by *Cochliobolus sps.* So we conclude that *Cochliobolus sps* isolated from plastic dumped soil in the present investigation has industrially important applications and is needed to be further subjected to strain improvement by mutation studies for the efficient cellulase production.

REFERENCES

Augusta, J., Muller, R.J., and Middeck, H. 1992. Biologics abbaubare Kunststoffe: Test verfahren und Beurteilungskriterien. *Chem Ing* Tech, 64: 410–415.

Bradner, J.R., Gillings, M. and Nevalainen, K.M.H. 1999. Qualitative assessment of hydrolytic activities in Antarctic micro fungi grown at different temperatures on solid media. World Journal of Microbiology and Biotechnology 15, 143–145.

Domsch, K.H., Gams, Wand Travate-Heidi, Anderson. 1980. Compendium of soil fungi, Vol.1 Academic press, London.

Ghosh, T.K. 1987. Measurement of cellulase Activities*. Pure and Appl Chem.* 59(2): 257.

Herr, 1979. Secretion of cellulases and β-glucosidases by *Trichoderma viridae* ITCC 1433 in submerged cultures on different substrates. *Biotechnology and Bioengineering* 21: 1361-1363.

Kawai, F. 1995 Breakdown of plastics and polymers by microorganisms. *Adv Biochem Eng/* Biotech 52:151–194.

Lekh Ram., Kuldeep Kuar., Sandeep Sharma 2014. Screening isolation and characterization of cellulase producing microorganisms from soil. *International journal of pharmaceutical science invention.* 3 (3): 12-18.

Lowry O.M., Rosebrough N.J., Farr A.L. Randall R.J. 1951. Protein measurement with Folin - phenol reagent. *Journal of Biological Chemistry*. 193: 265-275.

Lynd, L. R., Weimer, P.J., Vanzyl, W.H and Pretorius. I.S. 2002 Microbial cellulose utilization: fundamentals and biotechnology. *Microbial. Mol Biol.,* Rev. 66: 506-577.

Mandels M., Weber J. 1969. The production of celluloses In: Gould R.F. (ed) Cellulases and its application. *Advances in chemistry Series. American Chemical Society,* Washington, DC, pp. 391-414.

Miller G.L. 1959. Use of dinitrosalicyclic acid reagent for determination of reducing sugars. *Anal. Chem.* 31: 426-429.

Muller R. 2009. Reserve Forestall El Chore: Analysis de deforestations y strategies Para reducible. Santa Cruz de la Sierra, Bolivia: Foundation Nature.

Naveen Kumar, K.J. and Thippeswamy. B. 2013. Isolation and screening of potent cellulolytic fungi from areca nut husk waste. *Int .J.Curr Sci*.8:E125-132.

Pečiulytė D. 2007 Isolation of cellulolytic fungi from waste paper gradual recycling materials. Ecological 53, 11–18.

Sabir M. I., X. Xu, L. Li, 2009. A review on biodegradable polymeric materials for bone tissue engineering applications, J. Mater. Sci. 44 5713-5724.

Seymour, R. B., and Stahl, G. A. (1986) Block Copolymers, Tamkang University Press, Taipei, Taiwan.

Shimao, M., 2001. biodegradation of plastics. *Curr.Opin.* Biotechnology., 12: 242-247.

Teather R.M. and Wood P.J. 1982. Use of Congo red Polysaccharide interactions in enumeration and characterization of cellulolytic bacteria from the bovine rumen. *Applied and Environmental Microbiology* 43: 777-780.

****