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Screening of cellulolytic fungi and evaluation of biodegradation potentialities of a selected strain on organic solid waste

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Abstract

This study focused on the screening of cellulolytic fungi isolated from different sources including soil, humus, agricultural wastes and kitchen wastes. Fungal isolates were isolated from different samples on selective medium and 32 isolates were tested on congo red plate for primary screening. Eight fungal strains have good cellulase potential and showed considerable amount of clear zone. These isolates are identified as *Trichoderma virens*, *T. pseudokoningii, T. harzianum Aspergillus ficuum*, *A. niger*, *A. tubingensis*, *Rhizopus* sp. and *Fusarium* sp. Out of eight isolates *T. harzianum* had more cellulose hydrolyzing capability and showed better performance on TLC plate. The maximum reducing sugar (365 µg/ml), protein (355 µg/ml) and biomass production (711 mg) was observed in *T. harzianum*. The maximum CMCase and FPase were also recorded in *T. harzianum*. This potential cellulolytic strain was further used for biodegradation of OSW with different treatments. The highest volume loss (43.59%) and weight loss (20.31%) was observed in T_1 treatment using kitchen waste and *T. harzianum* which was greater than 87.01 % and 79.71 %, respectively to control. It indicates *T. harzinum* is a promising fungus and can be apply in enhancing of organic solid waste biodegradation process to compost.

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Introduction

Lignocellulosic biomass such as agricultural crop residues and other energy crops is the most abundant and renewable biopolymer on Earth (Fukuda *et al.*, 2009). These materials are composed of three types of polymers namely cellulose, hemicellulose, and lignin which are strongly engaged and chemically bonded (Zhang *et al.*, 2006). Numerous microorganisms are capable of decomposing cellulose, although only a few of these microorganisms produce significant quantities of extracellular enzymes to decompose cellulose (Sukumaran *et al.*, 2005). Fungi are a goup of microorganism that can degrade mixtures of heterogeneous substrates such as municipal solid waste, cattle manure, and agricultural and industrial wastes (Singh, 2006) rapidly and for this they possess a complex set of extracellular enzymes.

A number of different plate screening methods to identify cellulolytic or polysaccharide-degrading microorganisms have been described which are typically based on either the formation of complexes between polysaccharides and dyes, the solubility and gel-forming characteristics of polysaccharides, or the use of soluble or insoluble dye-labeled polysaccharides (Ten *et al.*, 2004). The intensity of the colored zone depends on several factors, including substrate and enzyme concentrations, and the catalytic properties of enzymes (Coman *et al.*, 2008). Various biological studies have been carried out to identify the major microbiological agents responsible for biodegradation. Today, environmental policies and regulation progress lead to the development of biodegradation processes to turn organic wastes into a valuable resource by potential microbes which could have practical application on biodegradation of organic solid waste. Thus the present research has been undertaken to screen out a potential cellulolytic fungus through conventional method and its bioconversion potentialities were evaluated on organic solid waste to prepare compost.

Materials and methods

Sample collection

For isolation of cellulolytic fungi a total of twenty six samples were collected from different types of cellulosic wastes such as soil (usually where the cellulosic materials were rotten), humus, agricultural and kitchen wastes. The samples were kept in sterile polythene bags and preserved in refrigerator at 4° C for further study.

Isolation of cellulolytic fungi

Agar plate method of Muskett, (1948) and dilution plate method of Brierley *et al* (1927) was followed for isolation of fungi. Fungi, those grew upon on selective medium (PDA with 1% CMC) were considered as cellulolytic fungi and selected for primary screening.

Primary screening of cellulolytic fungi

The selected fungal isolates were screened for their ability to produce cellulases complex following the congo red plate screening method. The isolates were grown on PDA medium supplemented with 1% CMC and incubated at 27°C for 5 days. After incubation the Petri plates were flooded with congo red solution (0.1%), and after 15 min the congo red solution was discarded, and the plates were washed with 1M NaOH solution allowed to stand for 15–20 minutes. The clear zone was observed around the colony when the enzyme had utilized the cellulose.

Identification of selected fungi

Identification of selected strains was made by macroscopic and microscopic characteristics of the isolates. The generic identity of each colony was recorded and identification up to species level was tried wherever possible, with the help of standard mycological books and manuals.

(Gilman, 1957; Booth, 1971; Subramanian, 1971; Ellis, 1971; 1976 and Alexopoulos, 1979).

Preparation of culture extract

The selected organism was inoculated in 100 ml PDB medium with 1% CMC and allowed to grow at 28°C with manual shaking for 7 days. When the organism was grown profusely, the culture medium was filtered and the filtrate was centrifuged at 10,000 rpm for 10

minutes. Then the supernatant was used as the crude enzyme solution for the experimental purpose.

Measurement of reducing sugar

The amount of reducing sugar in culture filtrate was measured by following Miller, (1959) method using dinitro-salicylic acid (DNS) reagent and measuring the absorbance at 550 nm in a spectrophotometer (Spectronic 21)) using glucose monohydrate as the standard. The reducing sugar produced in the reaction mixture was expressed by the amount of reducing sugar released/ml.

Estimation of protein

Protein was estimated by the method of Lowry *et al.*,(1951) measuring the absorbance at 660 nm in a spectrophotometer (Spectronic 21) using bovin serum albumin as the standard.

Confirmation of cellulase activity by TLC

Cellulase is the enzyme which release sugar from cellulosic substrate and the released sugar were identified by thin layer chromatography (TLC), following the method as described by Touchstone and Dobbins, (1978).

Biomass yield

Biomass produced by fungi in stationary method was taken. The filter paper containing biomass residue was dried in oven at 80°C for a constant weight and amount of biomass was calculated by subtracting the weight of filter paper.

Carboxymethyl cellulase activity (CMCase)

Endo-glucanase activity (CMCase) was determined by using 1% CMC as the substrate in 0.02M acetated buffer pH 5.2. The reaction mixture containing 2 ml of 1% CMC and 2 ml of enzyme extract were incubated for 2 hours at 45°C, after which the release of reducing sugar was measured by Miller, (1959) method using DNS reagent. One unit (IU) of enzyme activity was defined as the amount of enzyme released reducing sugar/ml/min.

Filter paper activity (FPase)

For assay of FP-ase activity, 2 ml of enzyme extract was added to 1 ml of 0.02M acetate buffer, pH 5.2 belong with 50 mg filter paper strip (Whatman no. 1, 1×6 cm) in a test tube and after incubation at 50 °C for 1 hrs, the reducing sugar released was estimated by Miller, (1959) method using DNS reagent. One unit of filter paper (FPU) activity was defined as the amount of released reducing sugar/ ml/min.

Biodegradation of organic solid waste (OSW)

Organic solid as kitchen waste and garden waste were used for biodegradation process. Kitchen wastes were collected from different garbage centre of RU Campus and garden wastes were from garden of RU with polythene bag and cut into 2-3 mm in size and weighed into 50 g and kept in 150 ml quantity bottle and tightened with cotton plug. The bottles with OSW were then autoclaved at 121°C and 1.05 kg/cm² pressure for 15 minutes.

The selected strain was cultured on PDA medium at 30° C for 48 hours. Then culture disc (5 mm diam.) was cut with cork borer and applied @ 6 culture discs / bottle. Control treatments were performed with no inoculation. Sterile thermometer was also pushed through cotton plug for each bottle. Then the bottles were sealed with para film and labeled. All the process was completed inside the running laminar airflow. The changes of odor, pH, temperature, volume loss (%) and weight loss (%) of decomposed organic solid waste were observed after 10 days interval up to 30 days. For measurement of volume loss (%) and weight loss (%) the following formula was used:

Volume loss (%) $= \frac{V - V_1}{V} \times 100$, where V is initial volume and V_1 is final volume.

Weight loss (%) $= \frac{W - W_1}{W} \times 100$, where W is initial weight and W_1 is final weight.

Statistical analysis of the data

The experiment was conducted by using completely randomized design with three replications. Analysis of the variance (ANOVA) was done accordingly and

significant differences among the treatments mean were identified by least significant differences (LSD) test at 5% level.

Results

Primary screening of cellulolytic fungi

Different samples were plated on selective medium (PDA with 1% CMC) and total thirty two fungal isolates were recovered for primary screening following congo red plate. On the basis of clear zone measurement results only eight isolates i.e. S_1/gr (90 mm), S_2/Bl (62 mm), S_4/gr (90 mm), S_9/Bl (65 mm), S_{14}/W (90 mm), S_{15}/Bl (66 mm), S_{19}/D-gr (90 mm) and S_{20}/W (85 mm) (Table 1 and plate 1) were selected for secondary screening.

Table 1. Plate screening of cellulose-degrading microbes isolated from different sources.

Strains	Source	Color of colonies	Diameter of clear zone (mm)
S_1/gr	Garden soil	Green	90*
S_1/W	Garden soil	White	32
S_1/Br	Garden soil	Brown	15
S_1/Br	Garden soil	Brown	15
S_1/Bl	Garden soil	Black	15
S_2/Bl	Humus	Black	62*
S_2/gr-Bl	Humus	Greenish black	32
S_4/gr	Strawberry field soil	Green	90*
S_4/W	Strawberry field soil	White	25
S_5/Bl	Pea field soil	Black	19
S_5/Bl	Pea field soil	Black	25
S_5/gr	Pea field soil	Green	12
S_5/gr	Pea field soil	Green	11
S_6/P	Soybean field soil	Pink	14
S_6/Y	Soybean field soil	Yellow	11
S_9/Bl	Green house soil	Black spore	65*
S_9/W	Green house soil	White	24
S_9/W	Green house soil	White	25
S_{14}/W	Rice straw	White	90*
S_{15}/W	Rotten brinzal	White	42
S_{15}/Bl	Rotten brinzal	Black	66*
S_{15}/Bl	Rotten brinzal	Black	56
S_{16}/Bl	Rotten capsicum	Black	22
S_{16}/Bl	Rotten capsicum	Black	20
S_{17}/Bl	Rotten pointed gourd	Black	25
S_{17}/Bl	Rotten pointed gourd	Black	28
S_{19}/D-gr	Rotten kidney bean	Dark green	90*
S_{20}/W	Rotten zinger	White	85*
S_{21}/Bl	Rotten bitter gourd	Black	15
S_{21}/Bl	Rotten bitter gourd	Black	12
S_{27}/Bl	Rotten lemon	Black	24
S_{27}/Bl	Rotten lemon	Black	22

N.B:*= Showing the selected strains.

These eight isolates were identified as *Trichoderma* virens (S_1/gr), *Aspergillus ficuum* (S_2/Bl), *T.* pseudokoningii (S_4/gr), *A. niger* (S_9/Bl), *Rhizopus* sp. (S_{14}/W), *A. tubingensis* (S_{15}/Bl), *T. harzianum* (S_{19}/D-gr) and *Fusarium* sp. (S_{20}/W) (Table 2).

Table 2. Identification of selected cellulolytic fungal isolates.

Isolates No	Macroscopic characteristics and texture	Microscopic characteristics	Identified as
S_{19}/D-gr	Dark green and dull green.	Mycelia creeping and non-septated hyphae. Erect, smooth, penicillately branched conidiophores. Phialospores were sub-globose to elliptical, smooth walled.	*Trichoderma harzianum*
S_4/gr	Whitish to pale green.	Hairy and flappy mycelia. Branched and dendroid conidiophores. Spores were pigmented, smooth, elliposide.	*T. pseudokoningii*
S_1/gr	Initially light green became deep grass green.	Soft and leathery mycelia. Conidiophores erect, smooth, penicillately branched. Phialides were flasked shaped, sub-globose to elliptical.	*T. virens*.
S_9/Bl	Colony velvety, wooly, whitish but later turned black with yellowish reverse side.	Conidial head globose, large, black, Vesicles globose shaped, sterigmata two series. Conidia globose and echinulate.	*Aspergillus niger*
S_{15}/Bl	Black brown shade slighty and high sporulation in centre.	Mycelia were compact, cylindrical, branched white submerged. Conidial head globose, vesicles globose, conidiophore smooth, long and coarse, thin walled. Sterigmata biseriate, conidia globose and margin rough.	*A. tubingensis*
S_2/Bl	Black with very dark purplish brown.	Mycelia were submerged in center, branched with granule. Conidial head radiate conidia globosely rough and thick walled. Conidiophores erect sinuous brown shades with thick wall and sterigmata biseriate.	*A. ficuum*
S_{14}/W	Colony hairy, creamy powdery growth that later turned black.	As Aseptate hyphae, unbranched sporangiosphores are from the foot of rhizoids that enlarged in a cup-shaped.	*Rhizopus* sp.
S_{20}/W	Fluffy creamy growth that later turned pinkish with a yellowish reverse side	Septate with branched conidiophore and oblong conidia	*Fusarium* sp.

Secondary screening of cellulolytic fungi

The cellulolytic potentialities of the selected fungi were analyzed by measuring of sugar spot on TLC plate (Plate 2). *T. harzianum* (S_{19}/D-gr) (+++) had more cellulose hydrolyzing capability and showed better performance on TLC plate. The selected eight strains were further tested for reducing sugar, protein and biomass production and the results were significantly varied ($p \leq 0.05$) with different isolates (Table 3). The maximum reducing sugar (365 µg/ml), protein (355 µg/ml) and biomass production (711 mg) was observed in *T. harzianum*. All the isolates showed different level of CMCase and FPase activity (Table 4). The highest amount of CMCase (63 IU/ml) and FPase (38 IU/ml) was recorded in *T. harzianum* which was significantly varied ($p \leq 0.05$) with other isolates.

Table 3. Amount of reducing sugar, protein and biomass production by selected strains.

Strain	Reducing sugar (µg/ml)	Protein (µg/ml)	Biomass (mg) (mg)
T. virens	321±0.51	300±0.38	500±0.22
A. ficuum	312±0.54	307±0.21	409±0.31
T. pseudokoningii	325±0.48	305±0.32	605±0.45
A. niger	310±0.50	340±0.28	581±0.36
Rhizopus sp.	271±0.52	295±0.36	395±0.41
A. tubingensis	332±0.44	345±0.31	617±0.55
T. harzianum	365±0.38	355±0.42	711±0.28
Fusarium sp.	280±0.58	298±0.35	601±0.42
LSD ($p \leq 0.05$)	6.556	7.349	14.847

Table 4. CMCase and FPase activity of crude enzyme of selected strains.

Strain	CMCase activity (IU/ml)	FPase activity (IU/ml)
T. harzianum	63±0.60	38±0.41
T. virens	57±0.71	33±0.52
A. tubingensis	61±0.52	35±0.48
T. pseudokoningii	55±0.66	30±0.38
A. niger	54±0.41	29±0.46
A. ficuum	49±0.36	28±0.50
Fusarium sp.	45±0.61	27±0.37
Rhizopus sp.	43±0.73	21±0.39
LSD ($p \leq 0.05$)	2.096	0.902

Bioconversion of organic solid waste (OSW)

For decomposition of OSW by using culture disc treatment of *T. harzianum*, no bad smell was emitted after 30 days but in case of control the bad smell continued even after 30 days. It was found that the color of treated OSW was changed into deep greenish brown, light blackish brown and deep blackish brown after 10, 20 and 30 days, respectively. The initial temperature was 29°C and rapidly rose to a peak of 32°C after 18-20 days of decomposing and continued until 25-28 days of decomposition, after which the temperature reduced to a level as it was in its initial stage (Table 5). The initial pH values were ranged from 5.82 to 6.01 but after 10, 20 and 30 days of decomposition it was gradually increased and reached at 6.18 to 6.98, 7.01 to 7.59 and 7.42 to 8.48, respectively (Table 5). The highest weight loss and volume loss was recorded as 20.31% and 43.59 %, respectively after 30 days of decomposition in T_1 treatment but in control it was 5.66 % and 4.12 % (Table 5).

Table 5. Changes in temperature, pH, volume loss (%) and weight loss (%) of OSW using culture disc of *T. harzianum* during bioconversion.

Treatment	Initial temperature and pH		Changes of characteristics of OSW after 10 days				Changes of characteristics of OSW after 20 days				Changes of characteristics of OSW after 30 days			
	Tem °C	pH	Tem °C	pH	Volume loss (%)	Weight loss (%)	Tem °C	pH	Volume loss (%)	Weight loss (%)	Tem °C	pH	Volume loss (%)	Weight loss (%)
T_1	29	6.01	29	6.98	11.79±0.42	5.55±0.13	32	7.59	28.72±0.24	12.11±0.25	29	8.48	43.59±0.31	20.31±0.13
Control-1	27	5.82	27	6.54	1.79±0.08	0.61±0.42	28	7.11	3.63±0.57	1.88±0.61	27	7.69	5.66±0.79	4.12±0.48
T_2	29	5.92	29	6.71	9.02±0.17	2.88±0.32	31	7.22	20.96±0.68	7.91±0.89	29	8.0	31.42±0.64	13.17±0.69
Control-2	27	5.92	27	6.23	1.68±0.28	0.48±0.02	28	7.1	2.99±0.12	1.63±0.42	27	7.42	4.67±0.38	2.93±0.61
T_3	29	5.89	29	6.64	10.92±0.92	1.76±0.36	31	7.52	27.94±0.94	10.99±0.92	29	8.18	40.35±0.35	17.33±0.29
Control-3	27	5.89	27	6.18	1.51±0.18	0.59±0.41	28	7.01	2.61±0.19	1.82±0.31	27	7.51	4.31±0.28	3.99±0.39

Here,

T_1=kitchen wastes +culture disc of *T. harzianum,* Control-1=kitchen wastes,

T_2 =Garden wastes + culture disc of *T. harzianum,* Control-2 =garden wastes,

T_3 = kitchen wastes+ garden wastes + culture disc of *T. harzianum,* Control-3 =kitchen wastes +garden wastes.

Discussion

In this study fungal isolates were recorded from different types of samples using selective media containing CMC which supported the growth of the cellulolytic fungi (Khalid *et al.,* 2006). These isolates were screened on congo red plate and measured clear zone which was strong evidence that the fungi produced cellulase in order to degrade cellulose (Ram *et al.,* 2014). After primary screening total eight fungal isolates selected and identified as *Trichoderma virens*, *Aspergillus ficuum*, *T. pseudokoningii* , *A. niger*, *Rhizopus* sp., *A. tubingensis* , *T. harzianum* and *Fusarium* sp. In earlier studies Khokhar *et al.*, (2012) screened seventeen fungal species isolated from different sources with 1% congo red and *T. harzianum, T. viride, T. koningii, A. japonicus, A. nidulans ver. dentatus P. lanosum, P. expansum and P. oxalicum* gave the highest cellulase activity. The above species were also isolated with different numbers and frequencies from various sources in many places of the world by several workers (Chandel *et al.*, 2013 and Sharma and Sumbali, 2014).

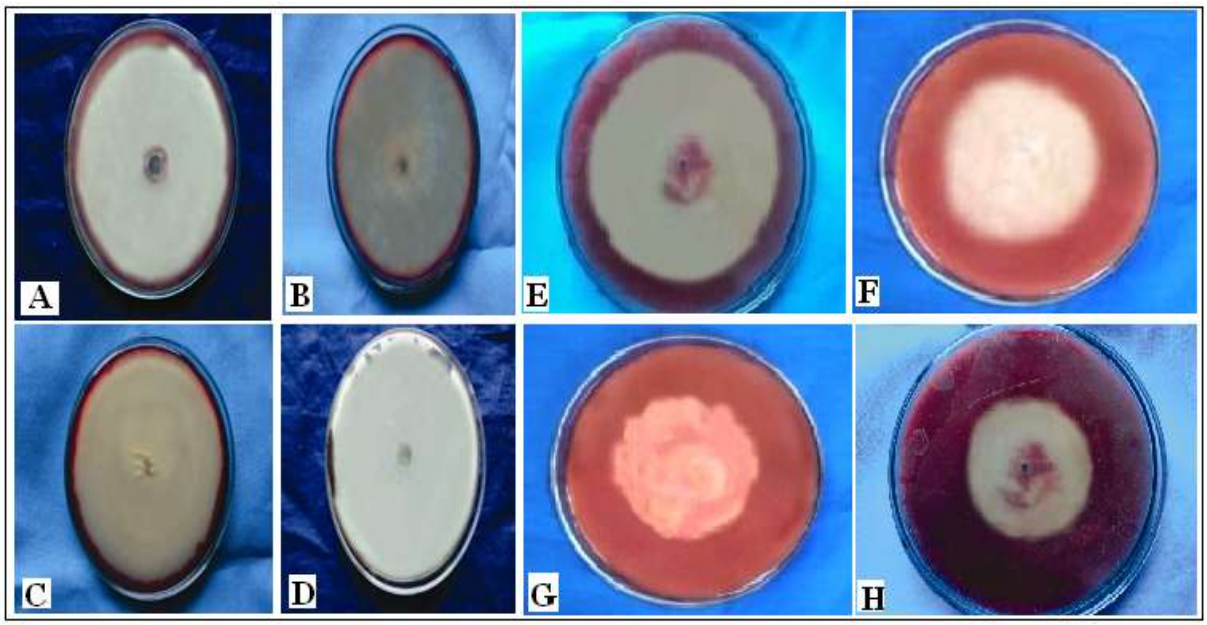

Fig. 1. Screening of fungal isolates for cellulose activity on congo red plate.

From TLC results it was observed that *T. harzianum* was highly active in hydrolysis of cellulase. Begum, (2006) studied comparatives cellulolytic activities of twenty four fungal strains by TLC plate method and observed that *A. oryzae*, *A. cervinus*, *A. flavus* str.3 and *A. ochraceus* were highly cellulolytic. Reducing sugar, protein and biomass production of eight fungal isolates were also evaluated and observed that *T. harzianum* was the most potent strain regarding these. In earlier studies it was observed that *Aspergillus fumigatus* (Rahman and Anwar, 1996) and *A. oryzae* (Begum, 2006) produce maximum amount of reducing sugar, protein and biomass.

Significant level of CMCase and FPase activity were exhibited in different isolates and *T. harzianum* showed maximum CMCase and FPase activities. The amount of CMCase was comparatively higher than FPase in all cases. Begum (2006) studied cellulase activity of *A. oryzae* and observed maximum 453, 393, 440 and 283 IU/ml of CMCase, avicelase, β-glucosidase and FPase activities, respectively. Sandhu *et al.,* (2009) reported *β*-glucosidase production by 39 fungi and found *A. fumigatus* and *A. nidulans* as good *β*-glucosidase producers. By comparing the earlier results of enzyme activity it was noticed that *T. harzianum* was the most potential cellulolytic fungi.

Degradation of organic solid waste is a complex process and requires participation of microbial cellulolytic enzymes. In our decomposition process after 30 days no bad smell was emitted in all

treatments but bad smell continued in controls which indicate the possible complete degradation of organic waste compare to control. After 30 days the mature OSW was deep blackish brown in color, granular, and fibrous with a pleasant earthy smell which indicated its maturity. Temperature is considered as a good indicator for the end of the biooxidative phase (Gautam *et al.,* 2010). The temperature levels in treatments and controls tended to increase due to the energy released from the biochemical reactions of the microorganisms. During the bioconversion of organic waste, there was a shift in pH from the initial condition acidic to alkaline condition. This increase in pH during the biodegradation process could be due to the production of ammonium as a result of the ammonification process (Huang *et al.,* 2004). Our results showed that the maximum pH of treatments reached to approximately 6.01 to 8.48 in T_1 treatment where kitchen wastes meet the compost regulations of pH 5.0–8.0 for the US, and pH 5.5–8.0 for the Council of European Communities (CEC) (Gautam *et al.,* 2011). The maximum volume loss and weight loss was also observed in same treatment which was 87.01% and 79.71 % greater than control. These findings are in accordance with the previous report of Rahman *et al.,* (2011) where volume loss and weight loss was 22.72% and 28.03%, respectively.

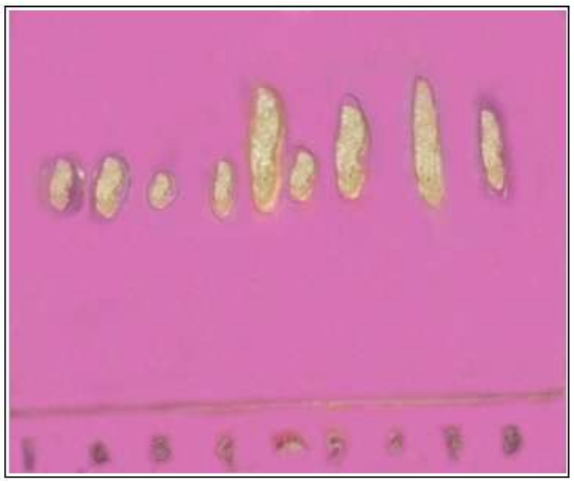

Fig. 2. Identification of cellulase enzyme by spot measurement on TLC plate.

Fungi are well known agents of decomposition of organic matter, in general, and of cellulosic substrate in particular (Lynd *et al.*, 2002). From above findings, it can be concluded that *T. harzianum* was the most potential cellulolytic fungi and had promising effect on the decomposition of OSW. Thus the strain may use to accelerate bioconversion process in rapid compost preparation.

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