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Characterization of fungi isolated from cattle dung with potential lignocellulolytic activities

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ABSTRACT

Agricultural wastes not only serve as sources of environmental pollutions, but also serve as magnificent source of plant nutrients and natural repositories of bio-tools especially fungi of industrial interest for lignocellulolytic enzymes production. However, residues management particularly in rice–wheat system is a tedious phenomenon due to narrow gap between harvesting and sowing of these two crops. Hence, microbial intervention is one of the key options that may use successfully for their management. Thus, cellulolytic fungi were isolated from the cattle dung collected from various locations for cellulose manufacturing. The cellulolytic property of fungal isolates was confirmed by plate screening assay based on yellow zone formation surrounding of fungal culture on Congo red agar medium plate owing to production of cellulase enzyme. The colony diameter, clear zone diameter and cellulolytic index showing in descending order of CRD3, CRD5, CRD6 and CRD10 isolates, but β–glucosidase activity was greater in CRD3 and CRD5 while, the lowest was in CRD9. However, isolates CRD7, CRD8 and CRD10 exhibited greater biochemical and enzymatic activity than rest of the promising fungal isolates. Similarly, isolates CRD1 and CRD2 utilized most of carbon sources as carbon substrate, but CRD7, CRD8 and CRD10 could not utilized mannitol and cellulose as carbon substrate. Overall, isolate CRD10 showed as a promising isolate in respect of biochemical, carbon utilization pattern and enzymatic activity than rest of the promising isolates.

Key words: *Cellulolytic index, Carbon utilization pattern, Dung, Enzymatic activity, Fungal isolate*

Introduction

Crop residues are very good sources of plant nutrients and producing stability of sustainable agricultural ecosystems. India generates 5500 Million tons of crop residue per year according to the Indian Ministry of New and Renewable Energy (MNRE) (NPMCR, 2019). In fact, majority of this crop residue is used as forage, fuel for other domestic and industrial purposes. However, there is still a surplus of 140 Mt, out of which 92 Mt is burnt each year (NPMCR, 2019). It is very interesting to note that the portion burnt as agricultural waste in India, its volume is much larger than the entire production of agricultural waste in other countries in the region. Besides use of mechanized harvesting and restriction on residues burning, farmers are still engaged in burning huge amount of crop residues in situ left in the field. Since, very narrow gap between rice harvesting and preparation of seed bed for wheat is sowing. That is the real causes of residues burning, and also there is no sound mechanism to decompose, incorporate and or remove crop residue from the field, to make field ready for timely wheat sowing. In addition, incorporation of rice straw before wheat planting compared to wheat straw before rice planting is difficult due to low temperature and the short interval between rice harvest and wheat plant-

ing. As crop residues left in the field, interfere with tillage and seeding operations for the next crop, hence, farmers often prefer to burn the residue in situ than removing from the field. Burning of crop residues in situ caused dropping of nutrients and organic matter in the soil. Unlike removal or burning, incorporation of straw builds up soil organic matter, soil N, and increases the total and available P and K contents of the soil. Apart from carbon, about 25% of nitrogen and phosphorus, 50% of sulfur, and 75% of potassium uptake by cereal crops are retained in crop residues, making them valuable nutrient sources. Region/area wherein mechanical harvesting is followed, a large quantity of crop residues is left in the field which can be recycled for nutrient supply. Both rice and wheat are nutrient exhaustive crop, depleting most of the macronutrients and micronutrients from the soil heavily. A rice-wheat sequence which yielded 7 tons ha^{-1} of rice and 4 t ha^{-1} of wheat liable to removes more than 300 kg N, 30 kg P, and 300 kg K ha^{-1} from the soil. The major disadvantage of incorporation of cereal straw is the immobilization of inorganic N and thus leads to N deficiency. Incorporation of cereal crop residues immediately before sowing/transplanting into wheat or rice significantly lowers crop yields. Generally, rice straw incorporation gave significantly higher wheat yields of 3.51 tons per ha compared to 2.91 tons per ha with straw removal. Incorporation of rice straw three weeks before wheat sowing significantly increases wheat yields on clay loam but not on sandy loam soil (Bijay-Singh *et al.,* 2004).

Cellulose is the most common ingredient of rice and wheat residues and is abundant in form of polysaccharide on the earth. Cellulose is renewable biopolymer on the earth and agriculture field, produces about 1.5×10^{12} tons of the total annual waste material by the photosynthesis process (Klemm *et al.,* 2002; Bhat, 2000). Cellulose is considered as one of the most important carbon source on this planet and its annual production occurs at a rate of 0.85×1011 tons per annum biosynthetically produce by both land plants and marine (Nowak *et al.,* 2005). Cellulose degradation and their subsequent utilizations are very important aspect for global carbon sources degraded through the cellulose enzyme. Thus, the value of cellulose is important for intense research and industrial interest because cellulose produce energy by hydrolysis of cellulose (Bhat *et al.*, 2000). Cellulose degradation carried out by cellulases enzyme, produced by different microorganism with higher specific activities and greater efficiency (Subramaniyan and Prema, 2000). In recent years, several microorganisms including some fungal strains played a vital role in cellulose-degrading, as cellulase enzyme liable for its decomposition have been isolated and studied extensively (Bhat *et al.,* 1997). Cellulolytic organisms such as fungi, bacteria, actinomycetes and protozoa play an important role in natural biodegradation processes in which plant lignocellulosic materials are efficiently degraded by cellulase enzymes. These enzymes have found novel applications in the production of fermentable sugars and ethanol, organic acids, detergents and other chemicals in industry. Cellulolytic enzymes that are mainly produced by diverse groups of microbes, but fungi and bacteria are main cellulose degrading (Lederberg, 1992). The biomass utilized by the aerobic and anaerobic mesophilic bacteria, filamentous fungi, thermophilic and alkaliphilic bacteria, actinomycetes and certain protozoa.

However, fungi are well known agents for cellulose degradation having cellulosic substrate in particular organic biomass (Lynd *et al.,* 2002). Because cellulose is the major component of plant biomass, which is mainly present in cell wall of plants, which produces about 4 ×109 tons annually by plants.

Cellulose is polysaccharides made up by thousand numbers of glucose units linked together by β-1, 4 glycosidic linkages. Cellulose has crystalline structure, and its insoluble nature represents a big challenge for enzymatic hydrolysis. Some microorganisms have the ability to convert lignocelluloses wastes into valuable products like biofuels, produced by fermentation process (Sánches *et al.,* 2009). Bioconversion of agricultural biomass material mainly depends on nature of cellulose, sources of cellulolytic enzyme and optimal conditions for catalytic activity and production of enzymes. Over the years, cellulose degrading fungi have been isolated and characterized for obtaining more effective cellulases from various sources such as soil, decayed plant materials, hot springs, organic matters, feces of ruminants and composts. The primary cell wall of green plants, many forms of algae and the oomycetes is composed of cellulose which is the structural component of cell wall, though some fungal species secrete it to form biofilms (Seneviratne *et al.* (2008). Cellulase is an enzyme liable to break down cellulose into smaller polysaccharides under cellulolysis or cellodextrins process in which cellulose completely break down into glucose units

through hydrolysis reaction. Apart from agriculture, the cellulose enzymes are mostly used in various industries such as textile industry for "bio-polishing of fabrics and producing stonewashed look of denims, household laundry detergents for improving fabric softness and brightness. The cellulases that are used for various industrial applications are isolated mainly from fungi (Tolan and Foody, 1999). In this study, we tried to isolate and characterize some promising fungal isolates from soil, contaminated with cattle dung, decomposed organic sources and to assess their efficacy of cellulolytic property by various bio-chemicals screening method.

Materials and Methods

Sample collection

Eighty decomposed cattle dung samples were collected from compost pit and open cow dung wards from different places of Lucknow, Uttar Pradesh. The samples were picked up with the help of sterilized plastic spoon and kept in sterilized polythene bags for further study. These samples were mixed thoroughly to increase homogeneity and processed within 48 hours for the isolation of cellulolytic fungi.

Isolation of cellulytic fungi

Serial dilution was prepared by using normal procedure and samples were prepared in test tube containing 104, 105 and 106 dilution (Aneja *et al.*, 2003). Sample were uniformly spread on solidified Czapeck-Dox agar plate containing 2.0 gL^{-1} sodium nitrate, 1.0 gL^{-1} dipotassium hydrogen phosphate, 0.5 gL^{-1} magnesium sulphate, 0.5 gL^{-1} potassium chloride, 0.01 gL^{-1} ferrous sulphate, 30 gL^{-1} sucrose as carbon source, 20 gL^{-1} bacteriological agar and 0.2 gL^{-1} chloramphenicol to inhibit bacterial growth. Plates were incubated at room temperature (28–30 ^{o}C) for 7 day. The fungal isolates were purified through repeated streaking on fresh Czapeck-Dox agar plates and pure cultures were stored on Czapeck-Dox agar slants and maintained in separate plate before testing their efficacy on selective media. These fungal inoculums were transferred to Carboxy methyl cellulose (CMC) plates for screening CMC activity, and incubated at 37 °C for 2 days. After that, these plates were flooded with 0.1% Congo red solution for about 20 min. and washed by using 1 M NaCl for 15 min. Formation of the clear halos zones around the colony is the indication that these fungal isolates have cellulose or cellulase degrading activity (Khianngam *et al.*, 2014). The yellow color zone formation directly related to cellulose utilization ability of fungal culture. The fungal colonies grown on the medium were sub-cultured on the Czapeck-Dox agar slants for further studies.

Screening of cellulolytic fungi

The cellulolytic property of fungal culture was confirmed by plate screening test. In this method, 1% of CMC (Carboxy methylcellulose) was amended with Czapeck-Dox agar media with pH 5.0 was poured in sterile petri dishes which solidify after some minutes. A small hole was put on centre of petri dishes aseptically wherein culture spores were added. After inoculation, plates were incubated for 3 days at 30 ^{0}C and 2 days at 50 ^{o}C. After incubation, the plates were stained with 1% Congo red solution for 15 minutes, after that the Congo red stain was neutralized with 1 M NaCl solution Carder JH. (1986). The yellow color zone formation showed the ability of cellulose utilization and enzyme activity of fungal culture.

Production of cellulases enzyme from fungal isolates

The isolated fungal cultures were tested to know their efficacy for cellulase production and its activities. A 100 ml Czapek-Dox broth amended with 1% cellulose was distributed into 250 ml separate conical flasks before maintaining 5.0 pH of the medium after which autoclaving at 121°C at 15 lb. pressure for twenty minutes. The fungal spore suspensions were inoculated into the conical flasks. The flasks were incubated at 32 °C on a rotary shaker at 120 rpm for 3 days. After 3 days, culture filtrate was collected, centrifuged at 6000 rpm for 15 min and supernatant was used for the estimation of extracellular cellulases enzyme.

Cellulase enzyme assay

Production of CMCase enzymes were measured by using CMC as the substrates by the methods suggested by Ghose (1987) and Ghose and Bisaria (1987) with slight modifications (0.1 M citrate buffer, pH 4.8 solution; 0.1 M citric acid and 1 M tri-sodium citrate). Reducing sugars produced due to enzymatic action were measured by 3, 5-dinitrosalicylic acid (DNS) method (Miller, 1959). The DNS solution was prepared by dissolving 10.6 g 3, 5-Dinitrosalicylic acid, 19.8 g sodium hydroxide, 306.0

Rochell salt (Na-K tartarate), 7.6 ml phenol and 8.3 g Na_2SO_3 and make up in 2 l water distilled water. This DNS reagent was stored in dark bottles at cool and dark places. Standard solution was made by dissolving one gram of CMC in 90 ml of 0.1 M citrate buffer (pH 4.8) and volume was makeup up to 100 ml in volumetric flask. Preparation of working solution, 10 ml of standard solution was taken in 100 ml volumetric flask and makeup volume up to 100 ml. Standard curve was prepared by taking 1, 2, 3, 4, 5, 6, 8 and 10 ml of the working solution in each 50 ml test tube and add 3 ml DNS solution in each tube and kept in boiling water bath for 5 minutes. After boiling, tubes were transferred immediately to a cold-water bath. After that add 20 ml distilled water and mixed properly by inverting the tube several times. The reducing sugars produced during this reaction were measured by using DNS method, at 540 nm in a spectrophotometer. The corresponding enzyme activity was calculated from glucose standard curve. The substrate blank was prepared by adding all the substances except, substrate solution. The enzymatic activity was expressed in International unit (IU) which defined as the amount of enzyme required to liberate 1 µmol of reducing sugars per min under the assay conditions.

The physical and biological tests were performed to assess the efficacy of isolated cellulose degrading fungi. Cellulase activity measured by using CMC agar plates poured with 0.1% Congo red reagent, left for 20 minutes after flooded with 1M NaCl (Henrissat, 1994). Chitinase activity was carried out by using Murthy and Bleakley (2012) methods, while ammonia production was determined through Cappucino *et al.* (1992) method. The IAA production and phosphate solublization were analyzed as per the method described by Bric *et al.* (1991) and Pikovskaya (1948) using spectrophotometer at 530 and 660 nm, respectively, whereas HCN production was determined according to Bakker and Schipper (1987) method. Xylenase activity was carried out by the method given by Azin *et al.*, (2007). Casein and starch hydrolysis was conducted as per method described by Cappuccino and Sherman (2008). Methyl red test carried out by Aneja, (2003) H2S production test carried out by Tille and Forbes, (2014).

Analysis of carbon sources

Different carbon sources such as glucose, sucrose, lactose, mannitol, cellobiose, cellulose, and xylose alone and in combination were analyzed by using Czapeck-Dox agar medium. The medium was inoculated with overnight fungal culture and incubated for 24 h at 37 ± 2°C /or 55 ± 2°C followed by taking OD through spectrophotometer at 540 nm according to the method of Miller (1959). Standard solutions of different concentrations of glucose, fructose, sucrose, lactose, mannitol, cellulose, and xylose were prepared by dissolving 2 mg ml^{-1} stock solution of glucose in 90 ml of 0.1 M citrate buffer (pH 4.8) and volume was made to 100 ml with distilled water. Out of which, 10 ml standard solution was taken in 100 ml volumetric flask and make up volume up to 100 ml with distilled water. A substrate blank (0.5 ml of glucose, fructose, sucrose, lactose, mannitol, cellulose, and xylose solution + 0.5 ml of citrate buffer) and enzyme blanks (0.5 ml of CMC solution + 0.5 ml of dilute enzyme solutions) were also prepared by adding all the substances except substrate which treat identically as the experimental tubes. Separate standard curve for each carbon sources were prepared by taking 1, 2, 3, 4, 5, 6, 8 and 10 ml of the working solution in 50 mL test tube and add 3 ml of DNS reagent. Tubes were boiled for 5.0 mm in a vigorously boiling water bath and after cooling in cold water bath after that added 20 ml of distilled water in each tube and take OD was at 540 nm. A standard graph of each carbon sources were plotted between concentration of glucose in X axis and absorbance at 540 nm in Y axis.

CMC media was used for assessing efficacy of cellulolytic fungi according to Mousdale (2008) in which cellulose degrading fungi produces halo zone around the colony due to production of cellulase enzymes. The efficacy of promising isolates was analyzed by measuring diameter of clear zone creates by various isolated and cellulolytic index was calculated by using formula as follows (Ferbiyanto *et al.*, 2015):

$$\text{Cellulolytic index (CI)} = \frac{\text{Clear zone diameter} - \text{Colony diameter}}{\text{Colony diameter}}$$

Results and Discussion

Identification of cellulolytic isolates

Colony diameter, diameter of hydrolysis zones of fungal isolates and cellulolytic index were used to assess the efficacy of various cellulolytic fungi. Out of ten promising cellulolytic fungi, CRD3, CRD5, CRD6, CRD8, CRD9, and CRD10 showed Congo-red staining colony diameter, clear zones diameter

and cellulolytic index (Nandana *et al.*, 2013). However, CRD1, CRD2 and CRD3 could not detect Congo-red staining colony diameter, clear zone formation and cellulolytic index. The highest colony diameter was recorded in CRD6 followed by CRD3, CRD8 and lowest in CRD5, whereas highest clear zone diameter was found in CRD6 followed by CRD3, CRD5 and CRD10. The cellulolytic index was highest in CRD6 followed by CRD3, CRD5 and least was in CRD8. These above promising isolates produce cellulase enzyme which degrade cellulose as stained with Congo red. Halo zone appeared around growing fungal colonies which indicate cellulose hydrolysis.

Table 1. Effect of different isolated promising fungal cellulose decomposing

Promising Isolates	Colony diameter (mm)	Clear zones diameter (mm)	Cellulolytic index
CRD1	ND	ND	ND
CRD2	ND	ND	ND
CRD3	17.0	4.0	13.0
CRD4	ND	ND	ND
CRD5	12.0	3.0	13.0
CRD6	21.0	7.0	15.0
CRD7	ND	ND	-
CRD8	15.5	1.0	10.7
CRD9	14.0	2.0	11.0
CRD10	15.0	3.0	12.0

*ND = Non Detective

The carbon utilization pattern was assessed in different cellulose degrading promising fungal isolates. Carbon utilization pattern was varied in different promising fungal isolates indicated that they had wide variations in their cellulose producing enzymes. Among the cellulose degrading fungal isolates utilized highest carbon in sucrose and followed in order of sucrose > glucose>xylose> dextrose> cellulose > mannitol (Fig. 3). Among the cellulose degrading fungal isolates, CRD6 was utilized highest glucose as carbon source followed by CRD2, CRD9 and CRD10 and least was utilized in CRD5. Contrary to that, mannitol and cellulose was utilized as carbon sources among the cellulose degrading in similar trends as CRD1 was utilized the highest mannitol and cellulose as carbon source followed by CRD2, CRD9 but CRD7 had negative effect on mannitol and cellulose as carbon source utilization. The sucrose content was utilized highest in CRD8 fungal isolates followed by CRD5, CRD2, CRD1 and least was utilized in CRD3. The dextrose was utilized highest in CRD3 followed by CRD7, CRD9, CRD4 and least was utilized by CRD8 whereas xylose was utilized the highest in CRD9 followed by CRD7, CRD3, CRD9 and lowest was utilized by CRD1. Lorliam *et al.* (2013) isolated 39 yeasts from the dung of various herbivores using xylose as carbon source.

Biochemical analysis

Biochemical analysis viz., β-glucosidase, chitinase, pectinase, xylanase and starch activity were performed to assess residues degrading activity of all the isolated promising fungal strains which indicated varied results. The highest positive β-glucosidase enzymatic activity was observed in CRD3, CRD4, CRD5 and CRD, while CRD6 fungal strains showed only positive in relation to β-glucosidase producing enzymes (Table 2). However, strains CRD1, CRD2, CRD7, CRD8 and CRD10 not detected β-glucosidase enzymatic activity. The highest chitinase activity was showed in CRD10, whereas

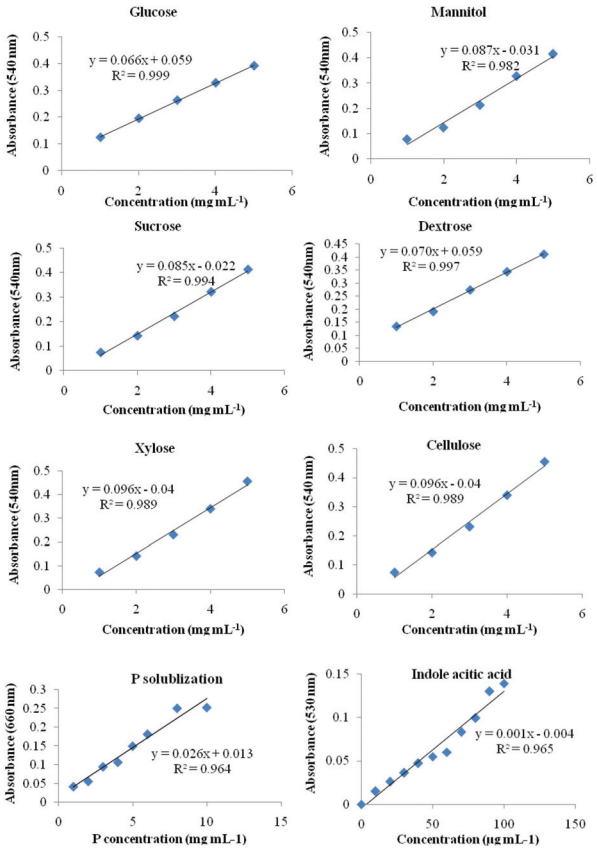

Fig. 1. Standard graphs of different carbon sources utilized by isolated fungal strains

CRD6 showed medium positive and CRD3, CRD4, CRD5, CRD7, CRD8, and CRD9 exhibited only positive and CRD1 and CRD2 exhibited negative in relation to chitinase activity. Strains CRD1, CRD2, CRD5, CRD6 and CRD9 not detected the pectinase activity, while strains CRD7, CRD8 and CRD10 had highly positive in relation to pectinase activity and strain CRD3 and CRD4 showed positive pectinase activity (Table 2). The starch producing activity was the highest in CRD7 and CRD8 whereas CRD3, CRD5 and CRD10 showing positive activity in relation to starch producing activity. Zylanase activity was the highest positive in CRD6, CRD7 and CRD10 but CRD8 in medium positive and CRD5 had positive. However, zylanase activities were not detected in CRD1, CR2, CRD3, CRD4 and CRD9. La-Grange *et al.* (2010) reported that spergilli produces various classes of enzymes involved in the hydrolysis of cellulose, namely endogluconases, exogluconases, including cello dextrinases, cellobiohydrolses and -glucosidases. Amylase production was the highest in CRD7 and CRD8, while CRD3, CRD4, CRD5, and CRD6 showed only positive in amylase production but CRD1, CRD2 and CRD7 could not detected the amylase production. Other growth promoting substances viz., ammonia, H2S and catalase were also performed in promising fungal isolates. The highest positive activity of ammonia was recorded in CRD8, CRD9 and CRD10 but CRD3, CRD4, CRD5 and CRD6 reported only positive but CRD1, CRD2 and CRD7 could not detect the ammonia production.

The wide variations were observed in β–glucosidase enzymatic activity in different fungal strains as grown in differed fungal growing media. Similarly, cellulase activity was also differed in different fungal strains. Maximum cellulase produced from fungal culture Mutant and native fungal cultures such as *Aspergillus niger* both on submerged and solid-state fermentation. Similar results were reported by Reddi Pradeep and Narasimha (2012). The highest β–glucosidase activity was recorded in CRD4 (22.5 mg mgl^{-1}) and CRD6 (11.0 mg mgl^{-1}) while, the lowest was in CRD9 (8.25 mg ml^{-1}) and CRD1 (6.25 mg mgl^{-1}) as growing in Luria broth and nutrient broth, respectively (Table 3). However, cellulose activity was differed to μ–glucosidase activity in different

Table 2. Biochemical tests of each promising fungal isolates

S. No.	CRD1	CRD2	CRD3	CRD4	CRD5	CRD6	CRD7	CRD8	CRD9	CRD10
β-glucosidas	-	-	+++	+++	+++	+	-	-	+++	-
Chitinase	-	-	+	+	+	++	+	+	+	+++
Pectinase	-	-	+	+	-	-	+++	+++	-	+++
Xylanase	-	-	-	-	+	+++	+++	++	-	+++
Amylase	-	-	+	-	+	-	+++	+++	-	+
Ammonia	-	-	+	+	+	+	-	+++	+++	+++
H_2S	+	+	-	+	+++	++	+	+	-	+++
Catalase	+++	+++	+	+++	+	+++	-	+++	+++	+++

+ = Positive; ++=Medium positive; +++=Highly positive

Table 3. Evaluation of different promising fungal isolates on β–glucosidase, cellulase, IAA and P-solubilization

Fungal Isolates	β–glucosidase enzymes (mg mL^{-1})		Cellulase enzyme (mg mL^{-1})	IAA (μg mL^{-1})		P-solubilization (μg mL^{-1})
	Luria broth	Nutrient broth		(-) Tryptophan	(+) Tryptophan	
CRD1	16.9	6.25	0.39	99.6	312.7	9.92
CRD2	20.3	10.9	0.29	375.0	188.8	8.04
CRD3	21.2	6.81	1.43	93.5	81.9	6.17
CRD4	22.5	10.5	0.49	364.2	131.9	11.6
CRD5	12.6	9.17	1.12	58.1	183.5	10.3
CRD6	21.4	11.0	0.36	65.8	198.8	15.5
CRD7	16.5	9.03	0.41	124.2	618.1	7.64
CRD8	15.8	8.81	0.41	94.2	461.9	10.4
CRD9	8.25	8.51	0.39	214.2	116.5	10.2
CRD10	9.47	10.6	8.79	186.5	85	6.83
CD(0.05)	1.05	0.60	0.11	10.10	16.30	0.69

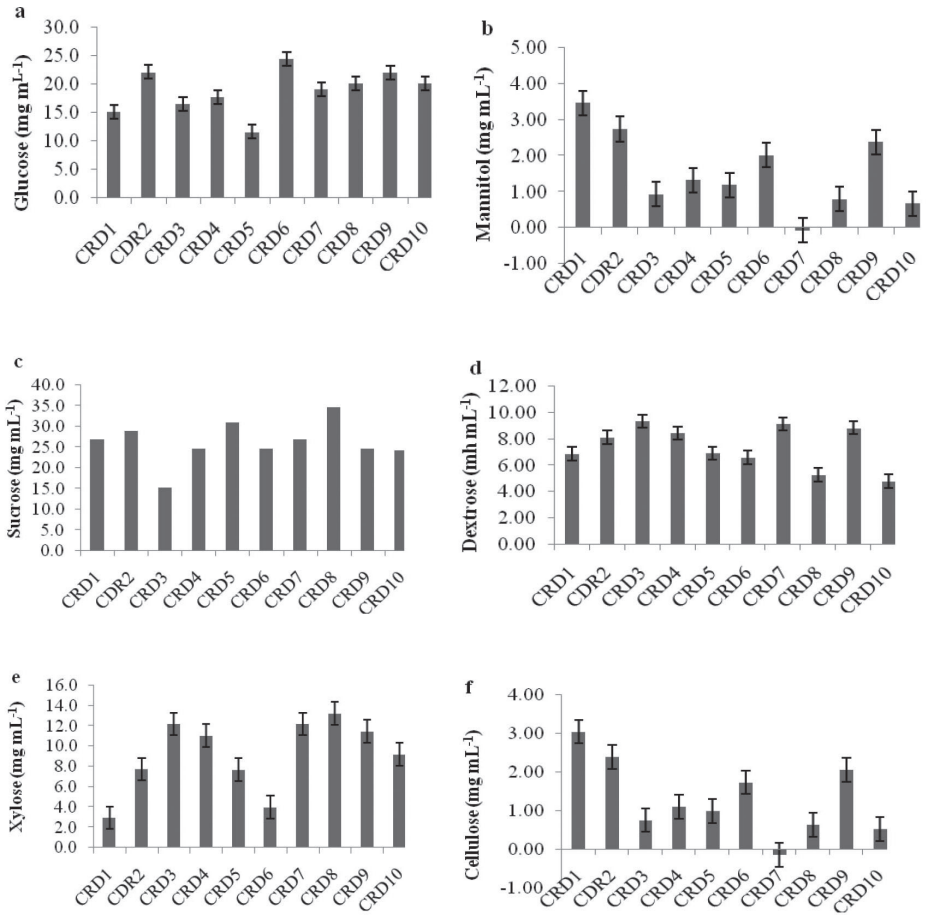

Fig. 2. Effect of different fungal isolates on carbon utilized pattern

promising fungal isolates, and the highest was in CRD10 (8.79 mg mgl^{-1}), but the lowest was in CRD2 (0.29 mg mgl^{-1}). Indole acetic acid was also differed as growing in different media. The highest indole acetic acid was recorded in CRD2 followed by CRD4 and CRD9 and the lowest was in CRD5 as growing with tryptophan media. However, CRD7 was recorded the highest IAA followed by CRD8, CRD6 and CRD2 as grown with tryptophan (Table 3). However, lowest IAA was observed in CRD5 and CRD3 when grown without and with tryptophan. The P-solubilization was the highest in CRD6 (15.5 µg ml^{-1}) followed by CRD4 (11.6 µg ml^{-1}) and CRD8 (10.4 µg ml^{-1}) and the lowest was in CRD3 (6.17 µg ml^{-1}).

Conclusion

Various fungal cultures were isolated from the cattle dung collected from different places of Lucknow and the cellulolytic activity of fungal culture was confirmed by plate screening assay method. The plate screening assay confirmed the cellulolytic property of fungal isolates. Production of cellulase, xylanase, chitinase, pectinase and amylase varies among the fungal isolates, except β–glucosidase activity. The cellulolyytic index was the highest IAA with tryptophan and P-solubilization was noted in CRD4. Overall, isolate CRD10 exhibited the highest cellulolytic activity as compared to rest of the fungal isolates.

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