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Evaluation of Various Agricultural Biomass for Cellulase Production by *Trichoderma* spp. under Solid State Fermentation (SSF)

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Abstract

The proximate and compositional analysis of fifteen agricultural biomass was done to evaluate their biotechnological potential. The analysis of ash, moisture, carbohydrate, crude protein and crude fibre along with cellulose, hemicellulose, and lignin content of selected local agro-lignocellulosic wastes was performed by using standard procedures. The ash content was found to be highest in rice husk (20.2 ± 0.02 %) and lowest in corn cob (2.52 ± 0.04 %). Moisture content was maximum in pea pod waste (8.67 ± 0.05 %) and minimum in groundnut shell (1.67 ± 0.05 %). The carbohydrate content was found maximum in the cotton stalk (58.12 ± 0.06 %) and minimum in soybean husk (1.12 ± 0.02 %). The maximum crude protein content was found in the green gram shell (12.64 ± 0.06 %) and the minimum in coconut coir (0.98 ± 0.02 %). Sugarcane bagasse (57.5 ± 0.80 %) showed the highest crude fibre whereas the lowest was reported in soybean stalk (7 ± 0.23 %). All the agro-residues showed a good amount of cellulose, hemicellulose and lignin. The highest cellulose and hemicellulose were seen in sugarcane bagasse (57.28 ± 1.2 %) and wheat straw (24.8 ± 0.01 %), respectively while both are reported as lowest in rice husk. The maximum lignin content was noted in rice husk (41.0 ± 0.09 %) and minimum in soybean husk (2.8 ± 0.08 %). Further, five agro-residues (banana peel, corn cob, groundnut shell, sugarcane bagasse and pigeon pea stalk) were used as substrates to produce cellulase by *Trichoderma harzianum* and *T. viride* under SSF. The results demonstrated that the assessed agro-residues can serve as an inexpensive feedstock for cellulase production.

Keywords: Lignocellulosic biomass, Solid state fermentation, Fungal cellulase, FPase, CMCase

In the twenty-first century, fossil fuel-based energy is non-feasible due to its cost, limited availability, and negative impact on the environment, which led to the exploration of economical and renewable energy sources (Caraschi *et al*., 2019). Amongst the several renewable choices, lignocellulosic biomass-derived biofuel has come up as the best substitute to fossil fuel since, these are cost-effective, abundantly available, and environmentally friendly (Yahya *et al*., 2015). However, the cost of cellulase enzyme needed for the biological transformation of these substrates into bioethanol which is one of the major impediments in the commercialization of bioethanol production from lignocellulosic biomass (Chovau *et al.,* 2013). In this context, utilizing lignocellulosic substrates for the production of cellulase enzyme can considerably lower the overall process cost. Several microorganisms are capable of producing the cellulases that can break the most abundant lignocellulosic compound, cellulose (Pachauri, *et al*., 2017).

Lignocellulosic biomass encompasses all vegetation including agricultural wastes, municipal residues, wood residues, and other residue materials (Ayeni *et al*., 2013). Cellulose, hemicellulose, and lignin cover the major chunk of lignocellulosic biomass. They are held together by various bonds and forces establishing a complex structure that contributes to the recalcitrance of the lignocellulosic biomass to enzymatic hydrolysis and insolubility in water (Menon and Rao, 2012). Apart from these major components, lignocellulosic biomass also contains other compounds such as water (moisture), a small amount of proteins, ash, organic acids, and minerals. High variability and uncertainty among lignocellulosic materials exist as their compositional characteristics vary (Kang and Tan, 2016).

The proximate analysis methods are used to determine the moisture content, total solids, volatile matter, ash, and the fixed carbon content of the substrate (Garcia *et al*., 2013). The compositional analysis of the lignocellulosic quantifies the structural components such as cellulose, hemicellulose, and lignin. Typically, the lignocellulosic biomass is made up of cellulose (40-50 %), hemicellulose (23-32 %), and lignin (15-25 %) (Sun *et al*., 2016). However, this composition of the lignocellulosic residues varies with the type and location of biomass, climate, and the nature of soil where they propagate (Yengkhom *et al*., 2017). Since the comprehensive and correct characterization of lignocellulosic biomass is a fundamental requirement for any process, it is necessary to characterize the selected local agricultural lignocellulosic biomass through the proximate and compositional analysis for assessing the potential of agro-residues as substrates for the production of cellulases employing Solid State Fermentation (SSF). After proximate and compositional analysis of fifteen agricultural residues, five of them with maximum cellulose content and low lignin content are assessed for their ability to produce cellulase using *Trichoderma harzianum* and *T. viride* employing SSF.

Material and Methods

Biomass Sampling and Preparation of Substrates

The collection of agro-residues was done randomly by taking their availability to the locality into consideration. For the present study, the selected agricultural biomasses are collected from the farms nearby the Parbhani district of the Marathwada region (Maharashtra, India). These agricultural substrates were banana peel, corn cob (maize cob), coconut coir, cotton stalk, cotton boll shell, soybean stalk, green gram shell (mung bean shell), rice husk, corn stalk (maize stalk), groundnut shell (groundnut husk), wheat straw, soybean husk, pea pod waste, sugarcane bagasse, and pigeon pea stalk (tur stalk).

The biomass residues were sun-dried under ambient conditions with utmost care that the samples were not over-exposed to sunlight. The sun-dried samples were powdered to a minimum particle size of 1 mm, sieved and stored in an air-tight container for further study.

Proximate analysis

The selected substrates were analyzed for ash, moisture, carbohydrate, crude protein, and crude fibre content.

Ash

Ash content of the biomass sample is the measure of the solid residue left after the substrate is burned. The oxide form of silica, iron, aluminum, calcium, sodium, potassium, magnesium, and titanium are the principal elements of ash (Vassilev *et al*., 2013). The ash content was determined by the method of Andrew and Agidi (2015) and the percentage of ash content was calculated as:

$$\text{Ash content (\%)} = \frac{W2}{W1} \times 100$$

Where, W_1 is the weight of the oven-dried sample and W_2 is the weight of the ash.

Moisture

Moisture content is the amount of water in biomass, which is expressed as a percentage of the material weight that affects all the processes associated with the substrate including the resultant products (Karunanithy *et al*., 2013). The moisture content (%) was determined by using the following equation (Andrew and Agidi, 2015).

$$\text{Moisture content (\%)} = \frac{(W1-W2)}{W1} \times 100$$

Where, W_1 is the weight of the sample and W_2 is the weight of the oven-dried sample.

Carbohydrate

The carbohydrate contents of samples were calculated by using the method of Hedge and Hofreiter (1962).

Crude protein

The protein content in the substrates was determined by AOAC (2004) method. The percentage of protein content in the substrate was computed using protein factor 5.7 as follows:

$$\% \text{ Nitrogen} = \frac{(\text{TS-TB}) \times \text{Normality of acid} \times \text{meq. } N_2}{\text{weight of sample}} \times 100$$

Where, TS is the titre value of the sample (ml), TB is thetitre value of the blank (ml), and Meq.N_2 is 0.014

$$\% \text{ protein} = \% \text{ Nitrogen} \times 5.7$$

Crude fibre

The crude fibre determination was made employing the method of Maynard (1970), which was calculated by the following equation:

$$\text{Crude fibre } (\%) = \frac{\text{Loss in weight on ignition (W2-W1) - W3-W1)}}{\text{Sample weight}} \times 100$$

Where, W_1 is the weight of the sample dish, W_2 is the weight of the oven-dried sample, and W_3 is the weight of the ash.

Compositional Analysis

In compositional analysis, cellulose, hemicellulose, and lignin content of the substrates were estimated.

Cellulose

The cellulose content in the sample was estimated according to the method of Gopal and Ranjhan (1980). The percentage cellulose concentration was calculated using the formula:

$$\text{Cellulose } (\%) = \frac{W2-W3}{W1} \times 100$$

Where, W_1 is the weight of the sample, W_2 is the weight of the oven-dried sample, and W_3 is the weight of the ash.

Hemicellulose

The hemicellulose content (%w/w) of dry biomass is calculated as per the method of Ayeni *et al*. (2013).

Lignin

The lignin content in the sample was measured by the procedure of Gopal and Ranjhan (1980). The percentage of lignin was calculated by the following equation:

$$\text{Lignin } (\%) = \frac{W2}{W1} \times 100$$

Where, W_1 is the weight of the sample and W_2 is the weight of the oven-dried sample.

Solid State Fermentation of Substrates for Cellulase Production

The SSF for cellulase production was carried out in commercial Petri plates by using two fungal cultures *T. harzianum* (MTCC 8230) and *T. viride* (MTCC 800). *Trichoderma* species were used in the present study for cellulase production owing to the fact that they are the most suitable cellulolytic candidates as compared to the other cellulase producing fungi like *Aspergillus* and *Humicola* spp. (Imran *et al*., 2016).

Five agricultural biomass residues *viz.*, Banana Peel (BP), Corn Cob (CC), Groundnut Shell (GH), Sugarcane Bagasse (SB), and Pigeon Pea Stalk (PPS) were chosen as substrates for estimating their potential to produce cellulase enzymes. SSF for cellulase production was carried out by taking five gram of each selected substrate and inoculating it with spore suspensions (10^8 spores/mL-suspension) of *T. harzianum* and *T. viride* at the loading of 0.1 mL per gram dry substrate in the separate sterile petri dish. The moisture content of all the substrates was adjusted to 70 per cent (wet basis) by adding sterile Mandel's media (Mandels and Weber, 1969) of pH five followed by incubation at 30 °C for 6 days under static conditions (Brijwani and Vadlani, 2011).

Cellulase Assay

After the extraction of enzyme as per the method of Brijwani and Vadlani (2011), the supernatant was collected and analyzed for Filter Paper (Total cellulase or FPase) activity and Carboxymethyl Cellulase activity (CMCase) using standard protocols described by Ghose, 1987. FPase activity was determined by mixing 1.0 ml of enzyme with 1.0 ml 50 mM citrate buffer pH 4.8 in a clean test tube. One Whatman filter paper strip (1.0 x 6.0 cm) was placed in the test tube containing the enzyme and buffer. The test tubes were then incubated at 50 °C in an incubator for 60 min. After the incubation, the released reducing sugar was determined by Dinitrosalicylic Acid (DNS) method (Ghose, 1987).

CMCase activity was determined by mixing 0.5 ml of 2 per cent carboxymethylcellulose (CMC) prepared in 50 mM citrate buffer pH 4.8 with 0.5 ml of the enzyme. The enzyme substrate mixture was incubated at 50 °C for 30 minutes and the reducing sugar produced was determined by DNS method (Ghose, 1987). One unit (U) of FPase and CMCase was defined as the amount of enzyme releasing 1 µmole of glucose from Whatman filter paper and Carboxymethyl Cellulose respectively per minute under standard assay conditions. The enzyme activity is expressed as Unit per mL (U/mL).

Results and Discussion

Biomass Sampling and Preparation of Substrates

The agricultural lignocellulosic biomass used in the study were collected from the local farms and were ground into powder form. The processed agricultural biomass substrates after crushing are shown in Fig. 1.

Fig. 1: Powdered agricultural biomass residues used in the study

Proximate Analysis

The results of proximate analysis of agricultural biomass are presented in Table 1.

Ash

The ash content of the fibrous feedstock is the inorganic elements such as salts of calcium, potassium, magnesium, and silicates present in it (Kumar *et al*., 2017). The maximum ash content of 20.2 ± 0.02 per cent was observed in the rice husk whereas corn cob showed the lowest ash content (2.52 ± 0.04 %) among all the agricultural substrates analyzed. These results slightly vary than those recorded by Cardoen *et al.* (2015) who reported ash content of 22.2 per cent and 1.2 per cent in Paddy husk and Maize cobs, respectively. The results for ash content in the present work conforms to those reported by He *et al*. (2014) who stated that ash is the measure of the total content of dust and inorganic constituents in biomasses, which is expected to be about 10 per cent in lignocellulose biomass. The ash content of BP, CC, GH, SB and PPS used in the present study for cellulase production was found to be 11.4 ± 0.42 per cent, 2.52 ± 0.04 per cent, 3.1 ± 0.21 per cent, 9.1 ± 0.3 per cent and 6.35 ± 0.43 per cent, respectively. These findings can be compared with studies of Pyar and Peh (2018) for BP; Cardoen *et al*. (2015) for Maize cobs, Groundnut shell and Sugarcane bagasse; Telang *et al*. (2010) for Tur straw.

Moisture

Low moisture content in substrates is essential for their enhanced shelf life as it will hamper the undesirable microbial activities in it. The lowest moisture content was observed in groundnut shell (1.67 ± 0.05 per cent) whereas maximum moisture content of 8.67 ± 0.05 is found in pea pod shell. The moisture content of analyzed agricultural substrates varied between 1.67 per cent to 8.67 per cent which showed that all the substrates were dried and stored well.

Carbohydrate

The carbohydrate content of the selected lignocellulosic substrates ranged between 1.12 and 61.2 per cent. Amongst all the substrates analyzed, the cotton stalk showed a maximum (58.12 ± 0.06 %) carbohydrate value while the soybean husk presented the minimum value of 1.12 ± 0.02 per cent. Other results include 23.4 ± 0.02 per cent, 46.18 ± 0.62 per cent, 22.2 ±

TABLE 1

Proximate analysis of agricultural biomass

Substrate	Ash (%)	Moisture (%)	Carbohydrate (%)	Crude Protein (%)	Crude Fibre (%)
Soybean husk	2.86±0.88	1.84±0.28	1.12±0.02	8.8±1.02	28.3±0.22
Cornstalk	8.46±0.32	2.94±0.16	34.88±0.12	3.08±0.12	41.12±0.83
Green gram shell	3.24±0.62	2.08±0.21	42.32±0.10	12.64±0.06	38.84±0.08
Corncob	2.52±0.04	2.96±0.14	46.18±0.62	3.86±0.38	31.52±0.08
Pea pod waste	4.86±0.03	8.67±0.05	51.12±0.06	9.88±0.04	8.14±0.04
Wheat straw	4.16±0.03	1.75±0.10	50±0.09	3.24±0.08	38.14±0.06
Banana peel	11.4±0.42	3.67±0.05	23.4±0.02	7.9±0.11	17.7±0.05
Coconut coir	2.60±0.04	6.93±0.07	46.12±0.09	0.98±0.02	33.12±0.06
Cotton stalk	4.88±0.12	5.2±0.10	58.12±0.06	11.3±0.39	18.7±0.07
Cotton boll shell	2.9±0.22	2.75±0.10	21.12±0.06	3.5±0.29	48.12±0.83
Groundnut shell	3.1±0.21	1.67±0.05	22.2±0.22	7.3±0.31	33.45±0.13
Sugarcane bagasse	9.1±0.3	3.96±0.14	46.4±0.11	2.8±0.08	57.5±0.80
Soybean stalk	7±0.14	3.82±0.10	24.11±0.11	5.0±0.03	7±0.23
Rice husk	20.2±0.02	8.19±0.06	16.3±0.09	3.0±0.08	40.5±0.33
Pigeon pea stalks	6.35±0.43	5.95±0.06	44.25±0.25	10±0.05	7.65±0.03

Results are mean + SD of triplicate analysis

0.22 per cent, 46.4 ± 0.11per cent and 44.25 ± 0.25 per cent, respectively for BP, CC, GH, SB and PPS. The finding in this research can be compared with the reported result of Pyar and Peh (2018); Abubakar *et al.* (2016); Cardoen *et al.* (2015) and Telang *et al.* (2010).

Crude protein

The crude protein contents of the agricultural biomass varied from 0.98 ± 0.02 per cent for coconut coir to 12.64 ± 0.06 per cent for green gram shell. The protein contents of other selected wastes were 7.9 ± 0.11 per cent, 3.86 ± 0.38 per cent, 7.3 ± 0.31 per cent, 2.8 ± 0.08 per cent and 10 ± 0.05 per cent for BP, CC, GH, SB and PPS, respectively. The obtained data for protein content of BP, CC, GH, SB and PPS showed slight variations with the reports of Cardoen *et al.* (2015).

Crude fibre

The lowest crude fibre content was recorded in soybean stalk *i.e.,* 7 ± 0.23 per cent while the highest *i.e.,* 57.5 ± 0.80 per cent was recorded in sugarcane bagasse. Crude fibres in substrates such as BP, CC, GH, SB, and PPS were found to be 17.7 ± 0.05 per cent, 31.52 ± 0.08 per cent, 33.45 ± 0.13 per cent, 57.5 ± 0.80 per cent and 7.65 ± 0.03 per cent. These values compare favorably with the works of Pyar and Peh (2018) for BP; Abubakar *et al.* (2016) for corn cobs; Abdulrazak *et al.* (2014) for Groundnut shell; Cardoen *et al.* (2015) for Sugarcane bagasse; Telang *et al.* (2010) for Pigeon pea stalk.

Compositional Analysis

The results of compositional analysis of agricultural biomass are presented in Table 2.

Cellulose

Cellulose is the largest portion of most plants that accounts for around 35-50 per cent of the total dry weight of physical plant biomass (Somerville *et al.*, 2010). The cellulose content of the agricultural biomass samples ranged from 26 to 57.28 per cent. Rice husk showed lowest cellulose of 12.0 ± 0.26 per cent. The cellulose content of BP, CC, GH, SB and PPS was found to be 37.9 ± 0.09 per cent, 37.68 ± 1.38 per cent, 35.7 ± 1.41 per cent, 57.28 ± 1.2 per cent and 30 ± 0.34 per cent, respectively. These results are in accordance with the previous studies of Pyar and Peh (2018) for BP; Cardoen *et al.* (2015) for CC, GH and SB; Telang *et al*. (2010) for Pigeon pea stalk. The results showed that the selected agricultural lignocellulosic biomasses are rich in cellulose.

Hemicellulose

Hemicelluloses are heterogeneous polymers that comprise around 15 - 35 per cent of the entire plant material. Taking the complexity in the structure of the cell wall of the plant components into consideration, hemicellulose stands next to cellulose (Althuri *et al.,* 2017). The hemicellulose value of all biomass evaluated in this study was within the range of the stated values. The highest hemicellulose content of 24.8 ± 0.01 per cent was recorded in wheat straw and the lowest in rice husk (7.2 ± 0.33 per cent). These values corroborated with those noted by Cardoen *et al.* (2015) where hemicellulose of 7.2 per cent was reported in paddy husk while wheat straw showed slightly higher hemicellulose content (30 %). Hemicellulose content of BP, CC, GH, SB and PPS used in the present study was 23.9 ± 0.08 per cent, 14.12 ± 0.86 per cent, 18.7 ± 0.88 per cent, 9.28 ± 0.8 per cent and 17 ± 0.16 per cent that can be compared with earlier studies of Cardoen *et al,,* 2015; Mythili and Venkatachalam, 2013.

Lignin

Usually, most of the agricultural biomass contains nearly 10 - 25 per cent lignin (Iqbal *et al.,* 2011). The lignin content of some of the biomass examined in the present investigation shows considerable deviation from the earlier statement. In the present study, soybean husk showed the minimum lignin value (2.8 ± 0.08 per cent), whereas rice husk biomass was found to have the maximum lignin value (41.0 ± 0.09 per cent). These findings can be compared with the lignin content reported by Cardoen *et al.* (2015) who reported the lignin content of 4.9 and 43 per cent, respectively for soybean husk and paddy husk. Amount of lignin content present in BP, CC, GH, SB and PPS used in the study was 9.0 ± 0.55 per cent, 10.62 ± 2.18 per cent, 25 ± 1.03 per cent, 12.20 ± 1.1 per cent and 18.2 ± 0.34 per cent that was lower than those reported in earlier studies of Cardoen *et al.,* 2015 and Mythili & Venkatachalam, 2013.

These variations in the values of agricultural biomass components exists because of the differences among species, tissues, and maturity of the plant, their growing conditions and techniques used for measurement (Barakat *et al.,* 2013).

Evaluation of Selected Agro-residues for Cellulase Production under SSF

Taking results of proximate analysis into consideration, the indigenous agro-residues such as BP, CC, GH, SB and PPS were used as substrates for cellulase production since they were having high cellulose and low lignin content. This is mainly because the substantial amounts of lignin would cover the cellulose portion making it inaccessible for the action of cellulolytic enzymes (Oberoi *et al*., 2010). Thus, although having maximum cellulose, substrates like Cotton stalk (41 ± 0.08 %) was not selected for the cellulase production study because it also showed high lignin content (30.8 ± 0.14 %) compared with the low lignin containing substrates like PPS and GH.

The results of cellulase production by *T. harzianum* and *T. viride* using selected agricultural biomass revealed that all the five agro-residues are potential substrates for induction of cellulolytic enzymes

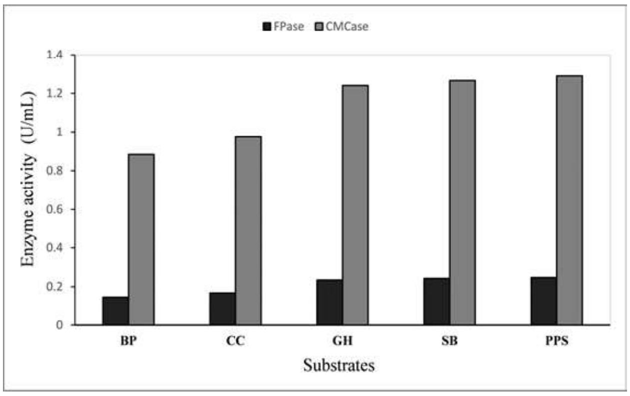

Fig. 2 Cellulase production by *T. harzianum* (MTCC 8230) under SSF of various agricultural biomass

TABLE 2

Compositional analysis of agricultural biomass

Substrate	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Soybean husk	31.64±0.42	11.58±0.14	2.8±0.08
Cornstalk	42.22±0.14	22.36±0.62	11.12±0.82
Green gram shell	26.26±0.46	14.36±0.05	9.21±0.42
Corn cob	37.68±1.38	14.12±0.86	10.62±2.18
Pea Pod waste	26.84±0.08	19.68±0.34	18.06±0.16
Wheat straw	36.8±0.04	24.8±0.01	15.22±0.03
Banana peels	37.9±0.09	23.9±0.08	9.0±0.55
Coconut coir	35.08±0.21	12.28±0.06	29.18±0.18
Cotton stalk	41±0.08	21±0.42	30.8±0.14
Cotton boll shell	48.7±1.38	18.5±0.82	22.3±0.62
Groundnut shell	35.7±1.41	18.7±0.88	25±1.03
Sugarcane bagasse	57.28±1.2	9.28±0.8	12.20±1.1
Soybean stalk	26±0.46	18±0.34	12±0.42
Rice husk	12.0±0.26	7.2±0.33	41.0±0.09
Pigeon pea stalks	30±0.34	17±0.16	18.2±0.34

Results are mean + SD of triplicate analysis

(Fig. 2 and 3). However, the maximum cellulase yield in terms of both FPase and CMCase was induced by Pigeon pea stalk followed by sugarcane bagasse, groundnut shell, corn cob and banana peels. Higher cellulase production with PPS and SB could be attributed due to more cellulose content compared to other three substrates (Table 2).

The results showed that PPS was the most effective substrate for cellulase production with FPase activity of 0.247 U/mL and CMCase activity of 1.291 U/mL by *T. harzianum* and FPase activity of 0.326 U/mL and CMCase activity of 1.586 U/mL by *T. viride.* SB was the next best substrate that showed 0.242 U/mL FPase and 1.267 U/mL CMCase activity by *T. harzianum*.

It is clear from Fig. 3 that after PPS, the second high cellulase production was obtained with SB with FPase of 0.323 U/mL and CMCase of 1.513 U/mL. This is in conformity with the results of Cunha *et al*. (2012) who stated that sugarcane bagasse is the best inducer of cellulase in fungi. The aim of employing these five substrates was to explore their ability to induce cellulase enzyme. From the results, it can be stated that all the five substrates are able to produce cellulolytic enzyme which is in agreement with earlier studies that reported the ability of these agricultural biomass to produce cellulase utilizing Banana peel (Sun *et al*., 2011); corn cobs, carrot peelings, composite,

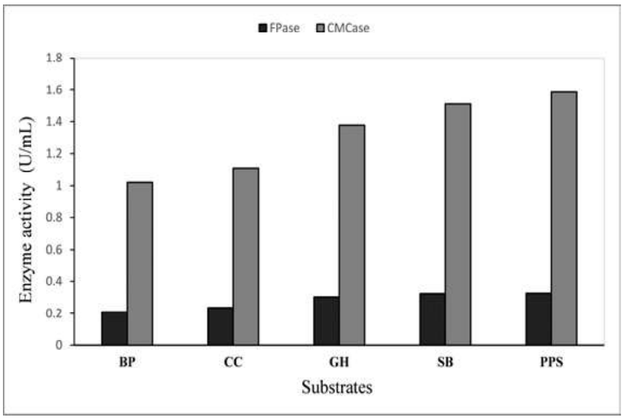

Fig. 3 : Cellulase production by *T. viride* (MTCC 800) under SSF of various agricultural biomass

grass, leaves, orange peelings, pineapple peelings, potato peelings, rice husk, sugarcane bagasse, saw dust, wheat bran, wheat straw (Bansal *et al*., 2012); Groundnut Husk (Salihu *et al*., 2013); Pigeon pea stalk (Kirti *et al*., 2019).

The prime objective of this work was to explore various raw agricultural biomass residues available in the Marathwada region (Maharashtra, India) for the production of cellulase enzymes. The selected lignocellulosic biomasses are highly indigenous to the Marathwada region of Maharashtra (India) and such exploration if added to the database of biomass can help in their utilization for future studies in areas like biofuel production. The resultant data from the proximate and compositional analysis of the selected local agricultural lignocellulosic residue is also an evidence for their potential to serve as a resource for future biofuel production. The present study also showed the potential of utilizing agricultural biomass for the production of cellulase by *T. harzianum* and *T. viride* which can be used to develop an economically viable cellulase production system. However, further studies in optimization of the process parameters for enhanced cellulase production are being considered.

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