International Journal of Botany Studies
ISSN: 2455-541X; Impact Factor: RJIF 5.12
Received: 07-04-2021, Accepted: 26-04-2021, Published: 30-04-2021
www.botanyjournals.com
Volume 6, Issue 2, 2021, Page No. 669-675

Fabrication and evaluation of field fermentor for ligno-cellulolytic enzyme production using biodigested slurry as a substrate

Manisha Parmar[1*], Jasvarinder Chalotra[2], Urmila Gupta Phutela[2]

[1] Department of Microbiology, College of Basic Sciences and Humanities, Punjab Agricultural University, Ludhiana, Punjab, India

[2] Department of Renewable Energy Engineering, College of Agricultural Engineering and Technology, Punjab Agricultural University, Ludhiana, Punjab, India

Abstract

The present study aims at fabrication and evaluation of 500L capacity fermentor for the production of ligno-cellulolytic enzymes using biodigested slurry as a substrate. The biodigested slurry has tremendous potential for the production of value added products, as it is rich in cellulose, hemicellulose and lignin. Enzyme production studies were carried out in fermentor utilizing two different fungal cultures namely *Humicola fuscoatra* MTCC 1409 and *Aspergillus* spp. The maximum versatile peroxidase activity of 0.204 Uml^{-1} and 0.140 Uml^{-1} was observed in *H. fuscoatra* MTCC 1409 and *Aspergillus* spp. after 6th and 8th day of fermentation, respectively. Maximum laccase activity of 0.045 Uml^{-1} was observed in *Aspergillus* spp. after 8th day of fermentation. Similarly, highest cellobiase activity of 47 Uml^{-1} was observed was observed in *Aspergillus* spp. after 6th day of fermentation.

Keywords: biodigested slurry, fermentor, *Humicola fuscoatra* mtcc 1409 and *aspergillus* spp., versatile peroxidase, laccase, cellobiase

Introduction

Microbial ligno-cellulolytic enzymes, well known as ligno-cellulases have received much attention latterly ascribed to their many industrial applications. These are characterized as a large group of extracellular biocatalysts, which comprises of lignin degrading enzymes, namely oxidases (laccase) and peroxidases (manganese and versatile peroxidase) and hydrolytic enzymes *viz*. cellulases and hemicellulases [1]. These enzymes facilitate the delignification of lignocellulosic substrates, depolymerization of carbohydrate polymers and fermentation of the sugars released, making them one of the major players in waste management [2].

Fermentation is a process which is performed in a vessel called bioreactor or fermentor, for the commercial production of enzymes and for carrying out important chemical transformations [3]. The dimensions of a bioreactor can vary, ranging from a microbial cell (measuring few mm) to shake flask (100-1000ml), to laboratory scale fermentor (1 to 50 liters), to pilot level (0.3 to10 m^3) and plant scale (2 to 500 m^3). Basic features of a bioreactor include headspace volume, agitator system, oxygen delivery system, foam control, temperature and pH control system, sampling ports, cleaning and sterilization system and lines for charging and emptying the reactor [4]. Batch bioreactors consist of a single tank with the potential of carrying out series of reactions and are provided with an agitator to intermix the substrate/ raw material. High yields of enzymes can be attained by employing submerged and solid-state fermentation utilizing inexpensive and easily accessible agro-wastes residues [5]. However, solid state fermentation sticks out in the mass production of ligno-cellulolytic and other important enzymes with diverse industrial applications [6].

Choosing an appropriate substrate for fermentation is a pivotal step as it is an important determining factor in the production of enzymes from microorganisms [7]. Lignocellulosic biomass is produced in gigantic amount annually and is considered as the most copious bio-renewable wastes on earth. These are not utilized to their full potential and bulk of the biomass ends up in landfills [8]. Biodigested slurry or bio-slurry, the by-product of anaerobic digestion technology, is rich in organic matter, macronutrients and bioactive compounds of low molecular weight and hence can be used as a substrate for production of value added products utilizing hydrolytic efficiency of filamentous fungi, actinomycetes and bacteria and better management of slurry along with controlling environmental pollution [9]. Concoction of cow dung and water (1:1) in the form of slurry is introduced in the bioreactor and bioconversion of this substrate into products like fermentable sugars, organic acids, fuel, single cell protein, amino acids, enzymes and industrial chemicals play a significant role in the economic viability of these value-added products.

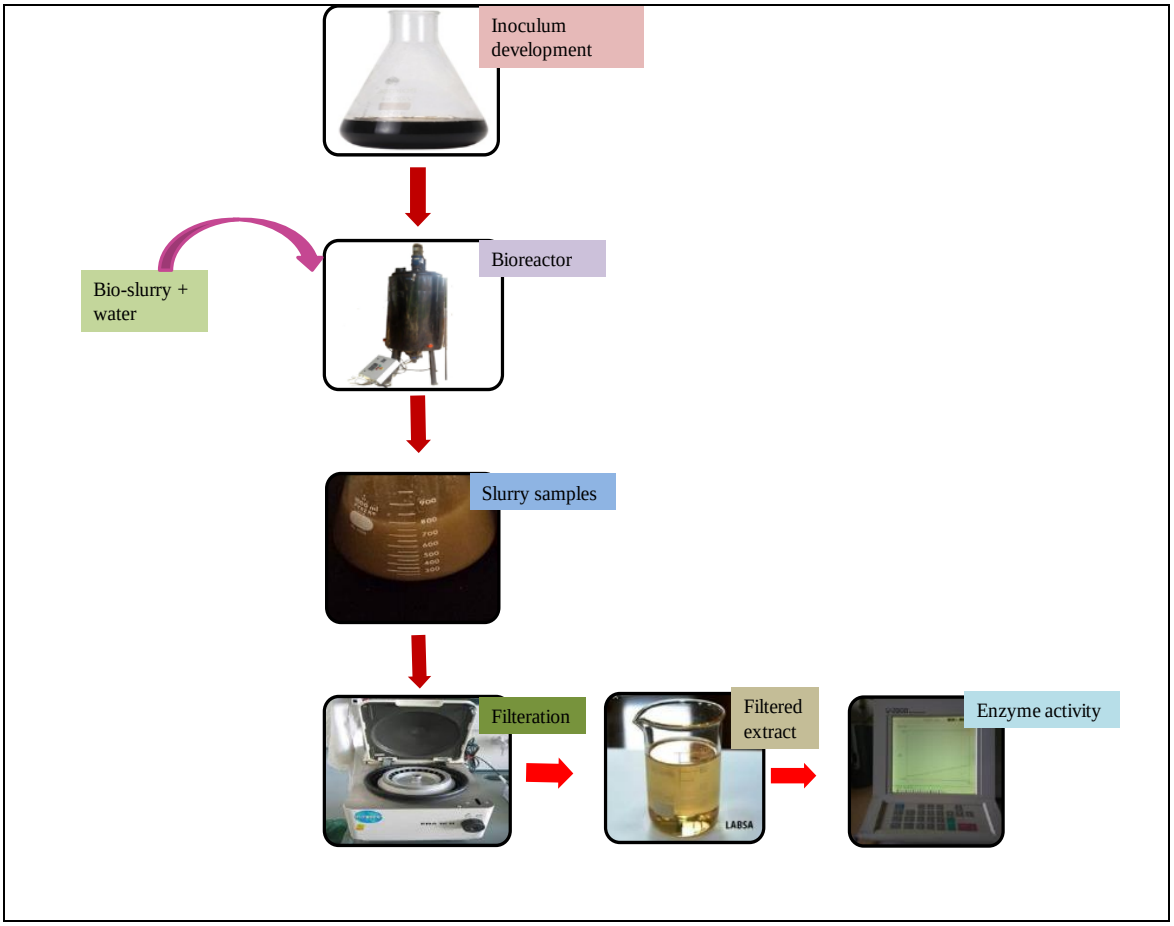

Fig 1: Industrial production of enzymes

Microbial enzymes have numerous industrial applications as in food, pharmaceutical, textile, leather and paper industry and are considered over conventional methods attributable to their cost effectiveness and greater efficiency. The schematic representation of industrial production of microbial ligno-cellulolytic enzymes has been shown in Fig.1.

Despite of many reports on the production of ligno-cellulolytic enzymes from the fungal species reports for up scaling of enzymes production using biodigested slurry is limited, therefore, the present study focuses on the fabrication and evaluation of a low cost fermentor for enzyme production and keeping in view the significance of bio-slurry as a possible substrate for enzyme production, the current study was undertaken to discern the production of ligno-cellulolytic enzymes from bio-slurry using fungal cultures. The objective of this work was to evaluate the production of different ligno-cellulolytic enzymes by *Humicola fuscoatra* MTCC 1409 and *Aspergillus* spp from biodigested slurry using the aforementioned fermentor.

Material and Methods

Fermentor: The fermentor (Fig.2), along with tripod stand, was made of mild steel**.** It was an assembly of three parts i.e., Electric heater, stirrer with geared motor and water jacket. The fermentor had a capacity of 500 lt, with dimensions of 3 feet x 5 feet. Three heaters, each having capacity of 3kw, were attached on its wall for heating and these heaters were capable of achieving a maximum temperature of 100°C. The heater had a display board and a thermo couple was placed in such a way that it can touch the inner wall of fermentor.

Fig 2: Liquid fermentor in working condition.

A stirrer of 5 feet long with 4 blades was attached on top of the fermentor to avoid settling of biodigested slurry and a speed controlled gear motor of capacity 1H.P was also attached to control its speed. Third part of the fermentor was water jacket that holds water and helps in transfering the heat in a controlled manner. Two mild steel drums were used in this design as a water jacket; one as inner drum and the other as outer drum.

The bioreactor was equipped with an inlet on the top of the bioreactor for feeding the material and an outlet was also

fitted out at the bottom for removing the material. It was also provided with a cone at the lower portion of drum to withdraw the sample easily. The whole fermentor was covered with an insulating sheet which avoids accidental chances.

Chemicals
All the chemicals utilized during enzyme estimation were of analytical grade and were purchased from SRL chemicals Pvt. Ltd.

Bio-digested slurry
Bio-digested slurry (BDS) for the production of ligno-cellulolytic enzymes was procured from a working biogas plant installed at demonstration area field of Department of Renewable Energy Engineering, PAU.

Microbial culture
Microbial culture of *Humicola fuscoatra* MTCC 1409, was procured from Institute of Microbial Technology, Chandigarh and was maintained on yeast peptone soluble starch media (YPSS; yeast extract = 0.4%, soluble starch = 1.5%, K_2HPO_4 = 0.1%, $MgSO_4$ = 0.1% and agar = 2.0%) agar slants. The culture was stored in refrigerator after sub-culturing at monthly intervals. *Aspergillus* spp. was isolated in the Biogas laboratory on malt extract agar. The culture was sub-cultured until pure culture was obtained. After the initial and microscopic examination, the culture was found to be *Aspergillus* spp.

Inoculum preparation
The foremost step for enzyme production is inoculum preparation. For *H. fuscoatra* MTCC 1409, ten liters of potato dextrose broth (PDB) was prepared, autoclaved and inoculated with 4mm bits of 7 days old culture. The flask was incubated at 45±2°C for 5 days.
For enzyme production studies, the fermentor was filled with a mixture of 5l biodigested slurry and 5l of water and was then inoculated with the 5 days old culture of *H. fuscoatra* MTCC 1409 (initially prepared in PDB) and was incubated for 5 days at 45±2°C. The fermentor was loaded with 150 litre of biodigested slurry and 150 litre of water. The BDS was inoculated with 10 litres of inoculum prepared in the lab and was incubated at 45ºC and 30 rpm. Similarly, the enzyme production by *Aspergillus* spp. was carried out at 30±2°C.

Estimation of lignolytic enzymes
About 50 ml of sample was removed regularly at an interval of 48 hours and crude enzyme was extracted by filtering the sample through filter paper Whatmann No.1. The filtrate was centrifuged at 10,000 rpm for 20 minutes at 4°C in a cooling centrifuge. The supernatant thus obtained was treated as crude enzyme extract and was used for estimation of laccase, versatile peroxidase, cellobiase and protein content [10]. The experiment was performed in triplicate. Enzyme activities (U ml^{-1} of sample) and protein (mg ml^{-1} of sample) was determined spectrophotometrically using UV-VIS spectrophotometer 2800 model.

Cellobiase assay
To 0.5 ml of enzyme extract, 0.5 ml of cellobiose solution was added and the mixture was incubated at 50°C for 10 min. Reducing sugar produced during this reaction was estimated using DNS [11].

Laccase assay
Laccase estimation was carried out according to the standard method [12]. Laccase activity was performed spectrophotometrically at 495nm in a reaction medium containing 1ml of the enzyme filtrate and 3ml of guaiacol solution. Change in absorbance was recorded for every 10 sec upto 180 sec. Enzyme activity was measured in Uml^{-1}, which is defined as the amount of enzyme required to oxidize 1µm of guaiacol per min.

Versatile peroxidase assay
Versatile peroxidase activity was determined by the standard method [13]. The manganese dependent activity of versatile peroxidase was determined by using manganese sulphate ($MnSO_4$) as substrate. The reaction mixture contained 0.2 ml of $MnSO_4$ (final concentration of 10mM), 2 ml of sodium tartarate buffer (pH 5) and 0.2 ml of the enzyme filtrate. The reaction was initiated by adding H_2O_2 (0.2 ml) and change in the absorbance was recorded for every 10 sec upto180sec at 238nm against a blank without H_2O_2.

Results and Discussion
Ligno-cellulolytic enzyme production from *Humicola fuscoatra* MTCC 1409: Scaling up of enzyme production was carried out in field fermentor of 500 L working volume. Samples were drawn from the fermentor at an interval of 48 hours for three weeks and were processed to study the enzyme production. It was observed that fermentation time has a marked effect on ligno-cellulolytic enzyme production. Results shown in Table 1 and fig.3 indicates that highest versatile peroxidase activity of 0.204 Uml^{-1} from *Humicola fuscoatra* MTCC 1409 was recorded on 6th day, followed by 0.123 U ml^{-1} on 4th day of fermentation. However, versatile peroxidase activity declined slowly afterwards and lowest enzyme activity was observed after 18th day of fermentation.

Table 1: Lignocellulytic enzyme production from *Humicola fuscoatra* MTCC 1409

Enzyme / Days	Versatile peroxidase (U ml^{-1})	Laccase (U ml^{-1})	Cellobiase (U ml^{-1})	Protein (mg ml^{-1})
0	0.040±1.73	0.021±3.21	4±0.57	0.74±2.11
2	0.041±1.52	0.033±1.52	6±1.52	0.75±1.76
4	0.123±1.73	0.025±1.73	13±1.15	0.96±1.73
6	0.204±2.64	0.023±2.88	24±1.73	1.05±2.04
8	0.121±1.52	0.015±2.88	17±1.52	1.38±3.55
10	0.111±3.78	0.013±0.58	9±0.58	0.48±1.45
12	0.109±1.73	0.011±0.58	5±1.52	0.46±2.12
14	0.108±1.52	0.008±1.15	0.90±0.01	0.36±2.03
16	0.075±1.73	0.007±1.73	0.20±0.02	0.29±2.31
18	0.051±1.52	0.004±1.15	0.05±0.02	0.24±1.45

Average of triplicate data, ± values indicates standard error
Cultural conditions: Temperature: 45±°C, pH: 5
Fermentation time is correlated to the availability of nutrients and other cultural conditions for the growth of microorganisms and production of enzymes. Generally, enzyme production is slow in the beginning of the fermentation and then it increases until a maximal value is attained, from there, the production decreases.

Laccase attained a maximum activity of 0.033 Uml^{-1} on 2nd day of fermentation (Table 1) and a sharp decline in activities was observed as the fermentation time increases. The lowest activity recorded was 0.0040 UmL^{-1} after 18th day of fermentation. Comparable effects of incubation time on enzyme activity were observed when large-scale production of laccase enzyme was conducted and submerged fermentation of *Cerrena unicolor* C-139 was carried out in a stirred bioreactor (120-L working volume) using wheat bran as the substrate. It was reported that maximum laccase activity of 416.4 UmL^{-1} was observed on 12th day of fermentation [14], which decreases with further incubation.

In an experiment carried out under laboratory conditions, the effect of incubation time on ligninolytic enzyme production from *Thermoascus aurantiacus* MTCC 375 using four different media specifically, Mandel's medium, biodigested slurry medium, diluted biodigested slurry medium and soaked paddy straw medium was studied. It was reported that maximal laccase activity of 785 U/g was observed in biodigested slurry medium [15]. High laccase activity in this medium could be due to the high content of lignin in digested biogas slurry.

Similarly, Cellobiase attained a peak activity of 24 Uml^{-1}on 6th day of fermentation, followed by 13U ml^{-1} on 4th day of fermentation. At the end of fermentation, the enzyme activity declined to attain a level of 0.05 Uml^{-1}. In an experiment carried out under laboratory conditions, Cellobiase enzyme production from *Humicola fuscoatra* MTCC 1409 was evaluated using paddy straw as a substrate in Mandel's media. Maximum cellobiase activity was recorded on 6th day of incubation and moreover, it was observed that further increase in incubation time did not show any increment in the enzyme production as laccase activity declined after 6th day [16]. This change in the level of enzyme production is ascribed to acclimatization of fungi at the start of fermentation, followed by utilization of media and nutrients for increased enzyme production. Eventually, there was a drop in enzyme production level which may be due to nutrient depletion and product accumulation.

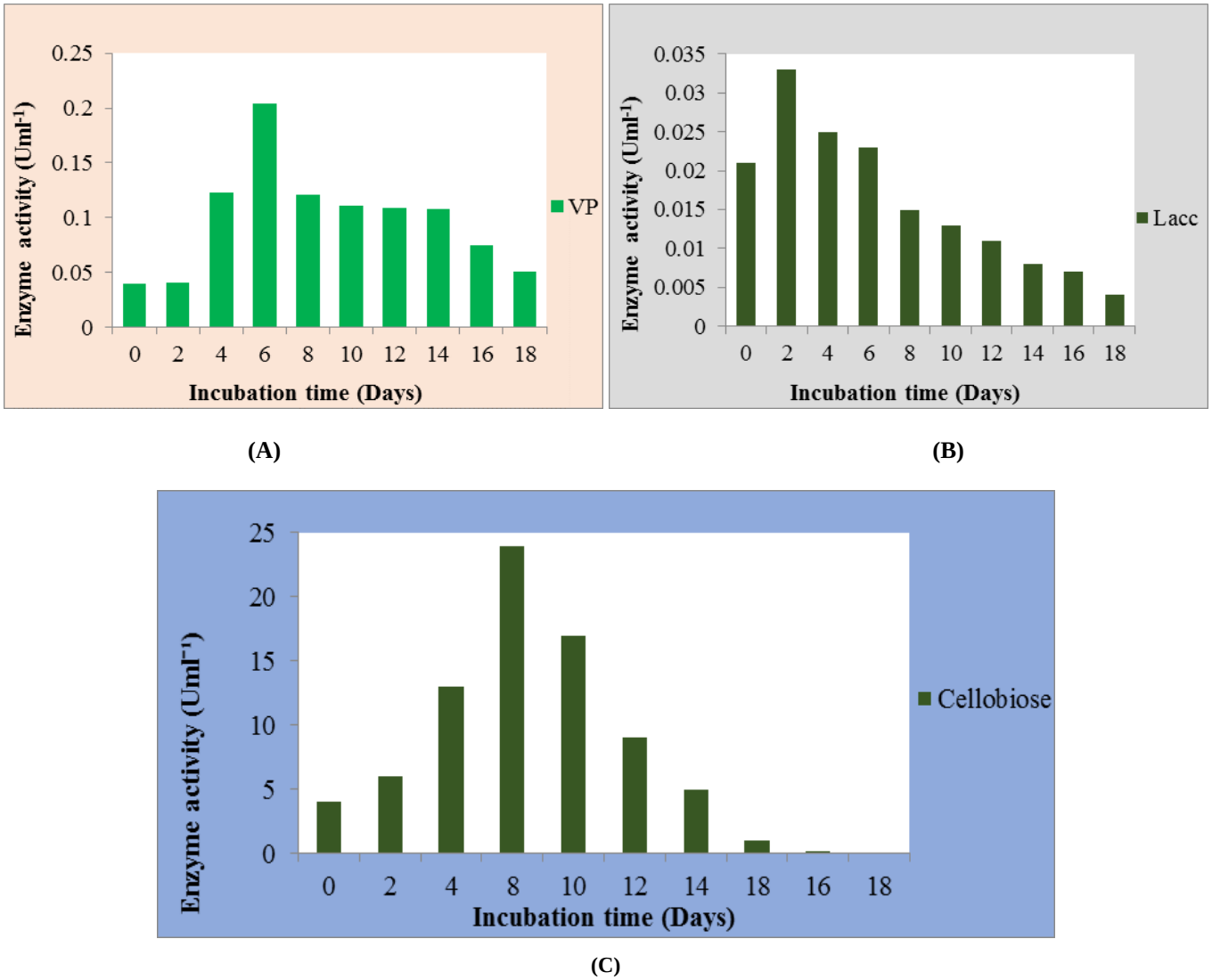

Fig 3: Production profile of (A) Versatile peroxidase, (B) Laccase and (C) Cellobiase from *Humicola fuscoatra* MTCC 1409

The induction of lignocellulytic enzymes from *Humicola* spp. depends on the kind of carbon source in the medium [17]. *Humicola fuscoatra* MTCC 1409 was a good producer of ligno-cellulolytic enzymes in a relatively inexpensive culture medium. Furthermore, crude enzyme extract contains sufficient levels of lignocellulytic enzymes and hence can be utilized to formulate a cost effective cocktail for lignocellulose hydrolysis.

A study was undertaken to optimize process parameters for production of cellulases by a thermophilic fungus Humicola

fuscoatra MTCC 1409 through solid state fermentation using paddy straw as a growth promoting Substrate. Response surface methodology was employed to optimize culture conditions for maximizing the Production of cellulases. Four different independent process parameters *viz*. temperature, pH, inoculum size and Incubation period were selected and effect of these parameters on production of different cellulases was ascertained.

Optimum conditions for production of cellulases from H. fuscoatra were temperature 45 ˚C, pH 6.0, Inoculum Concentration of 107 spores/ml and incubation period of 6 days A study was undertaken to optimize process parameters for production of cellulases by a thermophilic fungus Humicola fuscoatra MTCC 1409 through solid state fermentation using paddy straw as a growth promoting Substrate. Response surface methodology was employed to optimize culture conditions for maximizing the Production of cellulases. Four different independent process parameters *viz*. temperature, pH, inoculum size and Incubation period were selected and effect of these parameters on production of different cellulases was ascertained.

Optimum conditions for production of cellulases from H. fuscoatra were temperature 45 ˚C, pH 6.0, inoculum Concentration of 107 spores/ml and incubation period of 6 days

A study was undertaken to optimize process parameters for production of cellulases by a thermophilic fungus Humicola fuscoatra MTCC 1409 through solid state fermentation using paddy straw as a growth promoting Substrate.

Ligno-cellulolytic enzyme production by *Aspergillus* spp.: *Aspergillus* spp. utilizes variety of lignocellulosic substrates for the production of cellulose-degrading enzymes and Table 2 and fig. 4 represents the results of ligno-cellulolytic enzyme production from *Aspergillus* spp. It was observed that the maximum versatile peroxidase activity of 0.140 Uml^{-1} was recorded on 8th day, followed by 0.133 Uml^{-1} on 6th day of fermentation. However, its activity declined slowly afterwards.

Table 2: Lignocellulytic enzyme production from *Aspergillus* spp.

Enzyme / Days	Versatile peroxidase (U ml^{-1})	Laccase (U ml^{-1})	Cellobiase (U ml^{-1})	Protein (mg ml^{-1})
0	0.030±1.02	0.015±1.01	3±1.01	0.48±0.17
2	0.039±2.92	0.028±1.101	17±3.44	0.78±1.18
4	0.056±1.81	0.031±1.18	34±1.65	1.66±2.06
6	0.133±2.18	0.045±1.41	47±2.67	3.42±3.09
8	0.140±1.06	0.040±1.56	23±3.12	4.08±4.06
10	0.126±1.23	0.036±3.31	11±4.01	3.19±3.15
12	0.093±2.17	0.020±1.87	9±0.14	1.79±4.09
14	0.026±2.52	0.006±1.34	2±0.16	1.12±0.65
16	0.025±1.22	-	-	1.06±1.10
18	0.015±1.07	-	-	0.98±2.01

Average of triplicate data, ± values indicates standard error
Cultural conditions: Temperature: 30± °C, pH: 6.5

Aspergillus spp. has been extensively employed for the heterologous expression of ligninolytic peroxidases genes [18]. In a study the effect of temperature on versatile peroxidase production from recombinant *Aspergillus nidulans* was investigated using five discontinuous batch bioreactor cultures. It was reported that the maximal VP activity of 466 UL^{-1} was obtained after 115h at 19 ºC [19].

Laccase production utilizing various agro-horticultural substrates has been investigated by several researchers [20]. Table 2 indicates that the maximum laccase production was reached on day 6th day of fermentation with an activity of 0.045 Uml^{-1}, followed by 0.040 Uml^{-1} on 8th day and further increase in fermentation time reduces the enzyme activity. In the early stages, the laccase production was lower which increased later exponentially up to fourth to sixth days and then declined. The results were confirmed in a study wherein banana peel was opted as a substrate for the production of laccase enzyme from *Aspergillus fumigatus* VkJ2.4.5, it was reported that maximal laccase activity of 5,873.2 ± 34.07 U l^{-1} was detected after 6 days of fermentation, which declined to nearly 4000 U l^{-1} after 10 days of fermentation. The drop in the enzyme activity was attributed to the nutrient consumption and amassment of product [21].

In another study conducted on lab scale for the production of extracellular laccases by *Aspergillus flavus* isolated from natural habitat, it was observed that maximal laccase production of 17.39 $IUml^{-1}$ was observed at 12th day of fermentation [22]. The presence of water-soluble components of lignocellulosic biomass (such as *p*-coumaryl alcohol, coniferyl alcohol, sinapyl alcohol) stimulates the laccase production. Laccase genes have also been cloned and heterologously expressed in the filamentous fungi e.g., *Aspergillus niger*, *Aspergillus oryzae*, and *Trichoderma reesei* [23]. The recombinant enzymes share in general similar biochemical characteristics as the native ones.

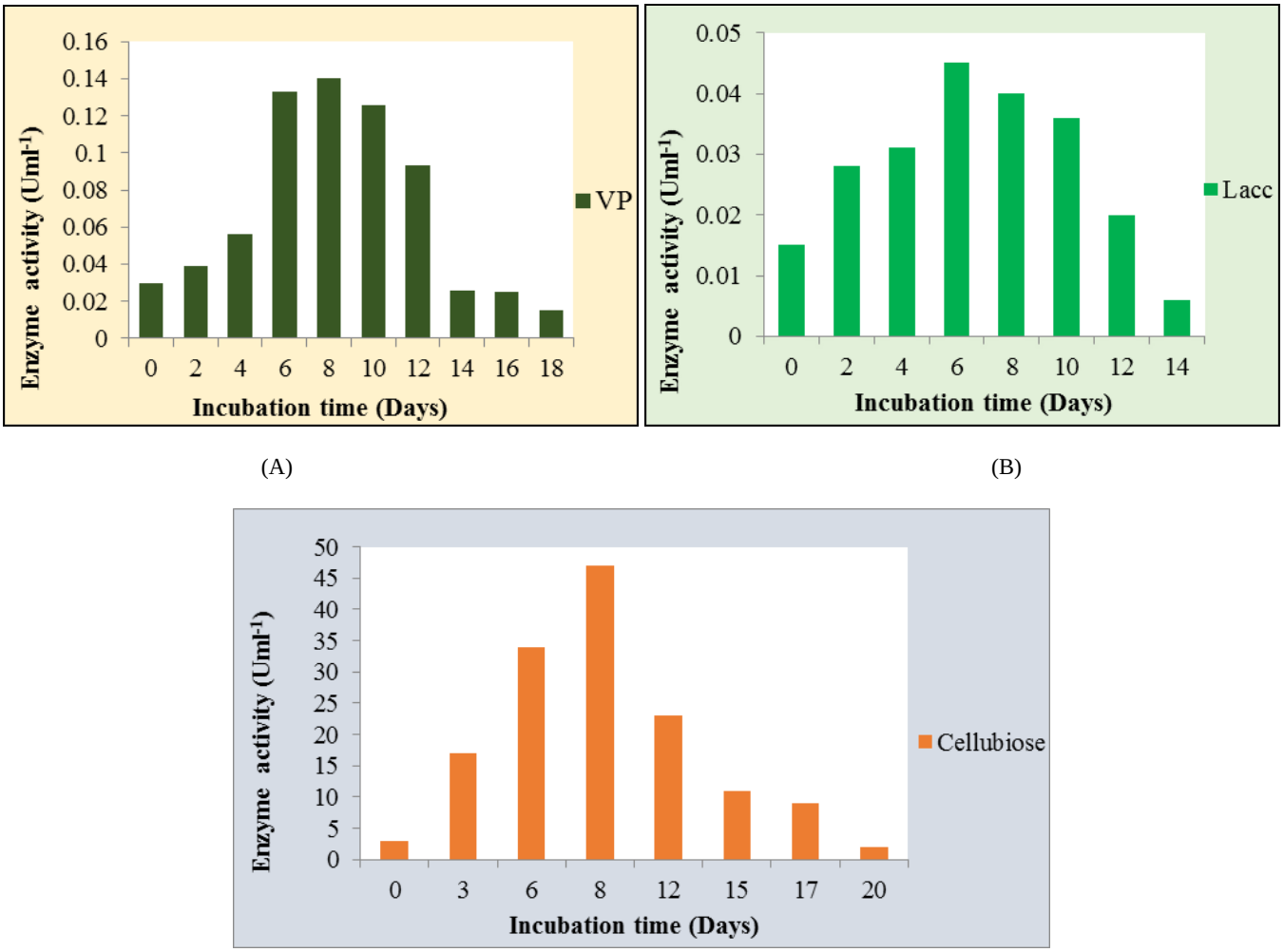

Fig 4: Production profile of (A) Versatile peroxidase, (B) Laccase and (C) Cellobiase from *Aspergillus* spp.

Similarly, cellobiase attained peak activity of 47 Uml^{-1} on 6^{th} day followed by 34 Uml^{-1} on 4^{th} day of fermentation. Similarly, cellobiase production was assessed in *Aspergillus niger* under solid state fermentation conditions using lignocellulosic substrates like corn cobs, rice husks and baggase. It was reported that baggase showed maximal cellobiase activity of 91.67 Uml^{-1} on the 6^{th} day of fermentation [24]. In a similar study carried out using *Aspergillus niger* NRRL3 for the production of cellobiase, moist wheat bran and ground corncob solid medium supplemented with inorganic minerals were used as growth medium. High concentration of cellobiase (215 IU/g of solid substrate) was reported by this method after 96 h of fermentation [25]. The activity of cellobiase is relatively low compared to endo- and exo-glucanases. As a consequence, improving the activity of cellobiase is pivotal in enhancing the saccharification yield of cellulosic resources.

Conclusion

Ligno-cellulolytic fungi and bacteria have attracted a great deal of interest for large-scale applications due to their ability to produce extracellular ligno-cellulolytic enzymes. Attempts are made to decrease the production cost of enzymes by using process integration. The problems of low yield, low productivity, and long residence time for microbial delignification and hydrolysis are the main challenge for scale up (industrial scale production). Therefore, isolation and identification of appropriate microorganisms, proper selection of raw materials (biomass), and optimization of process parameters (temperature, pH, aeration, particle size, time substrate concentration, and inoculum concentration) are compulsory for efficient utilization and conversion of the abundantly available resources.

References

1. Mtui GYS. Lignocellulolytic enzymes from tropical fungi: Types, substrates and applications. Scientific Research and Essays,2012:7(15):1544-55.
2. Nigam P, Singh D. Solid-State (Substrate) Fermentation systems and their applications in Biotechnology. Journal of Basic Microbiology,1994:34(6):405-23.
3. Singh J, Kaushik N, Biswas S. Bioreactors - Technology & Design Analysis. The SciTech Journal,2014:1(6):28-35.
4. Alaghlavi. Design of Fermenter and Kinetics. Bioprocess Engineering, 2013.
5. Couto SR, Toca HJL. Industrial and biotechnological applications of laccases: A Review. Biotechnology Advances,2006:24(5):500-13.
6. Seyma OE, Raziye OU. Production of ligninolytic enzymes by solid state fermentation using *Pleurotus ostreatus*. Annals of Agrarian Science,2017:15(2017):273e277274.
7. Goyal M, Phutela UG. Protease Production from Biodigested Slurry of Biogas Plants: A Review.

International Journal of Current Microbiology and Applied Science,2018:7(1):369-79.
8. Chen H, Liu J, Chang X, Chen D, Xue Y, Liu P *et al*. A review on the pretreatment of lignocellulose for high-value chemicals. Fuel Processing Technology,2017:160(1):196-206.
9. Suman V, Parmar M, Phutela UG. Evaluation of poultry dropping based biodigested slurry as a substrate for the cellulolytic enzymes production using *Penicillium roqueforti* NCIM 712. The Pharma Innovation Journal,2018:7(4):787-91.
10. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with Folin-phenol reagent. Journal of Biological Chemistry,1951:193:265-75.
11. Ghose TK. Measurement of cellulase activities. Pure and Applied Chemistry,1987:59(2):257-68.
12. Kalra K, Chauhan R, Shavez M, Sachdeva S. Isolation of laccase producing *Trichoderma* spp. and effect of pH and temperature on its activity. International Journal of Chem Tech Research, 013:5(5):2229-35.
13. Susana Camarero S, Sovan Sarkar S, Francisco Javier Ruiz-Duen FJ, Martınez MJ, Martınez AT. Description of a Versatile Peroxidase Involved in the Natural Degradation of Lignin That Has Both Manganese Peroxidase and Lignin Peroxidase Substrate Interaction Sites. The Journal of Biological Chemistry,1999:275(15):10324-330.
14. Songulashvili G, Spindler D, Jimenéz-Tobón GA, Jaspers C, Kerns G, Penninck MJ. Production of a high level of laccase by submerged fermentation at 120-L scale of *Cerrena unicolor* C-139 grown on wheat bran. Comptes Rendus Biologies,2015:338(2):121-125.
15. Phutela UG, Dar RA. Lignolytic enzyme production by *Thermoascus aurantiacus* MTCC 375 under solid state fermentation. Journal of Research PAU,2014:51(2):185-88.
16. Kaur B, Bhatia S, Phutela UG. Production of cellulases from *Humicola fuscoatra* MTCC 1409: Role of enzymes in paddy straw digestion. African journal of Microbiology Research,2015:9(9):631-38. 15.
17. Matsumoto H, Koganei K, Nishida N, Koyama Y Y, Saito S, Kataoka H *et al*. Cell dispersion culture for the effective growth of *Humicola insolens* and efficient enzyme production. Journal of Bioscience and Bioengineering,2014:117(3):257-62.
18. Larrondo LF, Lobos S, Stewart P, Cullen D, Vicuña R. Isoenzyme Multiplicity and Characterization of Recombinant Manganese Peroxidases from *Ceriporiopsis subvermispora* and *Phanerochaete chrysosporium*. Applied and Environmental Microbiology,2001:67(5):2070-75.
19. Eibes G M, Lu´-Chau TA, Ruiz-Duenas FJ, Feijoo G, Martınez MJ, Martınez AT *et al*. Effect of culture temperature on the heterologous expression of *Pleurotus eryngii* versatile peroxidase in *Aspergillus* hosts. Bioprocess and Biosystems Engineering,2009:32(1):129-34.
20. Zeng XK, Cai Y, Liao X, Zeng X, Li W, Zhang D. Decolorization of synthetic dyes by crude laccase from a newly isolated *Trametes trogii* strain cultivated on solid agro-industrial residue. Journal of Hazardous Materials,2012:187(1-3):517-25.
21. Vivekanand V, Dwivedi P, Pareek N, Singh RP. Banana peel: a potential substrate for laccase production by *Aspergillus fumigatus* VkJ2.4.5 in solid-state fermentation. Applied Biochemistry and Biotechnologym,2011:165(1):204-20.
22. Kumar R, Kaur J, Jain S, Kumar A. Optimization of laccase production from *Aspergillus flavus* by design of experiment technique: Partial purification and characterization. Journal of Genetic Engineering and Biotechnology,2016:14(1):125-31.
23. Piscitelli A, Pezzella C, Giardina P, Faraco V, Sannia G. Heterologous laccase production and its role in industrial applications. Bioengineered Bugs,2010:1(4):252-262.
24. Aliyah A, Alamsyah G, Ramadhani R, Hermansyah H. Production of α-Amylase and β-Glucosidase from *Aspergillus niger* by solid state fermentation method on biomass waste substrates from rice husk, bagasse and corn cob. Energy Procedia,2017:136:418-23.
25. Tsao GT, Xia L, Cao N, Gong C. Solid-state fermentation with *Aspergillus niger* for cellobiase production. Applied Biochemistry and Biotechnology,2000:84(86):743-9.