ENHANCED PRODUCTION OF LIGNOCELLULOLYTIC ENZYMES BY BACTERIAL CULTURE OF *DELFTIA* SP. THROUGH SUBMERGED STATE FERMENTATION OF BIODIGESTED SLURRY AND UTILIZATION FOR BIOGAS AUGMENTATION USING PADDY STRAW

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The bioconversion of various agricultural residues can be used to produce biofuels, biological products and biogas augmentation. The objective of this study was to produce lignocellulolytic enzymes by *Delftia* sp. PP4_S3 using biodigested slurry and their utilization for degradation of paddy straw for biogas augmentation. *Delftia* sp. PP4_S3 in biodigested slurry produced exoglucanase activity of 0.036 U mL^{-1}, β-glucosidase activity of 1.459 U mL^{-1}, manganese peroxidase activity of 0.244 U mL^{-1} and lignin peroxidase activity of 0.111 U mL^{-1}. RSM mediated optimization gave maximum enzyme activities at 50% slurry concentration, 1% inoculum and incubation period of 5 days. After upscaled submerged state fermentation, the paddy straw supplemented with enzyme enriched biodigested slurry showed 35% increase in biogas production, as compared to the control. A decrease in cellulose, hemicelloses and lignin percentage, with an increase in ash and silica percentage, was reported. Thus, *Delftia* proved to be an excellent source for lignocellulolytic enzyme production using biodigested slurry waste and subsequent biogas augmentation.

Keywords: lignocellulolytic enzyme production, bacterial culture, *Delftia* sp. PP4_S3, submerged state fermentation, biodigested slurry, paddy straw and biogas augmentation

INTRODUCTION

Paddy straw is a lignocellulosic biomass, which is a renewable source of energy and can be degraded by microorganisms. It has the potential to be used among other agro-wastes for biogas production and serves as a platform for a sustainable and environmentally friendly alternative to non-renewable sources of energy, such as coal, petroleum, fossil fuels and natural gas.[1] Energy production via renewable resources, like solar, wind, biofuel and biogas, reduces the carbon footprint and decreases the emission of greenhouse gases, which have a pronounced effect on climate change.[2] India produces 500 million tons of agricultural residues each year, as reported by the Ministry of New and Renewable Energy (MNRE). Uttar Pradesh (60 Mt) leads Punjab (51 Mt) and Maharashtra (46 Mt) in production of agro-wastes. The cumulative production of the three states contributes to the 500 Mt agro-residues reported each year by National Policy for the Management of Crop Residues (NPMCR). The large proportion of these agro-wastes undergoes industrial processes, and is used as fuel and fodder. As these crops have large returns for the farmers and the composition of paddy straw is too complex to be degraded by microorganisms, thus farmers are left with no alternative other than burning. Hence, there is a need to produce stable lignocellulolytic enzymes that can degrade paddy straw.

In the last few decades, biogas has gained importance as an alternative to conventional sources of energy, particularly in developing countries such as China and India. According to MNRE, 70% of population relies on biomass, which fulfills 32% of the energy needs of the country. India generated 2.07 billion cubic meters of biogas in 2018-2019, which corresponds to 5% of LPG expenditure.

Biogas consists of methane (50-60%), carbon dioxide (38-40%), nitrogen (0-1%) and other trace gases like hydrogen sulfide, carbon monoxide (0-2%) and water vapours.[3] Biogas is produced by 132,000 digesters of variable sizes in the world to generate electricity for supply into the electricity grid, enhancing the energy certainty from domestic sources, decreasing the reliance on fossil fuels, generating heat for cooking purposes and generating biomethane for vehicle fuel. The International Renewable Energy Agency (IRENA) statistics showed a 90% rise in electricity production from biogas, bringing out the transition from 46,008 GWh in 2010 to 87,400 GWh in 2016. Various physical, chemical and biological pretreatment methods are available in the literature for enhancing the biodegradability of paddy straw. Several studies have been conducted in the Biogas Laboratory of Punjab Agricultural University (PAU) to increase the biogas production by physical, chemical and biological pretreatments.[4,5]

Submerged state fermentation (SmF) has been used extensively in the past several years for the production of industrial enzymes and secondary metabolites due to its strict control of fermentation parameters, consistent productivity and easy downstream processing. The advantage of SmF over solid state fermentation (SSF) includes better heat and mass transfer rates, better diffusion of microbes and ease of commercialization at large scale, whereas high heat builds up in SSF and it is difficult to scale up in controlled parameters. However, high energy consumption, high cost of medium and complexity in operation make it an undesirable process.[6] The biodigested slurry constitutes 90-93% water, 7-10% of dry matter, which includes both organic and inorganic matter. It contains high nutrient content, including undigested organic matter, nitrogen (N), phosphorous (P), potassium (K), an array of macro- and micro-nutrients, such as zinc, manganese, magnesium, calcium, *etc*., and a variety of amino acids, along with solid residues. The nutrients in biodigested slurry are readily available, as a result of which they are used as fertilizer and act as a soil conditioner.[7] The cattle dung produced by animals in India is 730 Mt per annum. One kg of cattle dung can produce 0.3 Kg slurry. The total amount of slurry produced in India is 76.8 MT/year, which is drained in large amounts in the fields. Therefore, it is not feasible to carry it to farther places for physical and biological conversion, and it must occur in the vicinity of its origin. Many studies show that it can act as a cheap substrate for the production of lignocellulolytic enzymes, which can be further utilized for increasing biogas production.[5]

Lignocellulolytic enzymes act on lignocellulosic materials, such as paddy straw, and include cellulases, hemicellulases, and lignolytic enzymes that are produced by wood rot fungi, filamentous fungi, certain bacteria, and actinomycetes *etc*. Among fungi, the class Basidiomycetes are the most prominent ones as they are capable of mineralizing lignin efficiently.[8] Various fungi producing lignolytic enzymes are *Aspergillus*, *Gleocladium*, *Trichoderma*, *Coriolus versicolor*, *Geotrichum* and many more. White-rot fungi produce various isoforms of extracellular lignolytic enzymes. The extraction of enzymes using bacteria is more cost-effective due to their rapid growth, production of multi-enzyme complexes, with higher functionality and specificity. They are able to tolerate a wide range of stress conditions and allow better uncoupling of lignin from cellulose. Several genera involved in lignocellulolytic enzyme production are *Pseudomonas*, *Cellulomonas* and *Streptomyces*.[9] A recent study reported the isolation and optimization of enzymes using paper cup-based vermicompost and observed 45% degradation by bacterial consortia in three months. The paper cups were mixed with cattle dung in suitable proportions, which would transform into a vermicompost in a short period of time.[10] One study reported using *Bacillus megaterium* MYB3 bacteria for the decomposition of lignocellulosic biomass of corn stover and rice straw.[11] The decomposition rate of cellulose and hemicelluloses in rice straw was 44.87% and 29.32%, respectively. Other research work identified the BMC-9 strain of bacteria, which shows great ability to rapidly degrade the lignocellulosic residue of rice straw under relatively inexpensive conditions.[12] Recently, bacterial enzymes, such as laccases and DyP-type peroxidases, have been discovered to play an important role in lignin modification or its decomposition.[13]

Delftia species are gram-negative, rod-shaped, motile, non-sporulating, oxidase and catalase positive, non-pigmented bacteria, belonging to the Comamonadaceae family, which may occur singly or in pairs. The genus *Delftia* have been isolated from different environments, like fresh and marine water, clinical samples, infected plants

and activated sludge.[14] Some studies have reported on the ability of *Delftia* sp. isolates to fix N_2, produce phytohormones to be used as biofertilizers.[15,16] *Delftia* sp. are also considered as a good agent for cleaning of contaminated environments.[17] They have been characterized by their ability to transform or degrade multiple organic pollutants, including aniline, chloroniline, linurin and diuron.[18] An increase in biogas production by anaerobic digestion of corn stalk was reported by the lignolytic bacterial strain *Enterobacter hormaechei* KA3. The biogas production increased by 20% for KA3 inoculation, compared to the control group.[19] Another study reported that the application of lignolytic bacteria *viz. Agrobacterium* sp., *Paenibacillus* sp. and *Comamonas testosteroni* increased methane production and degradation of oil palm empty fruit bunches. Lignin breakdown of 25% was reported by *Lysinibacillus sphaericus*, with the highest methane potential of 0.042 m^3/kg.[20] Also, bacterial lignolytic enzymes produced by decaying wood samples showed maximum enzyme activity response by isolate L15, among other sixteen isolates, which grew on all lignin monomers and can be used to degrade various classes of dyes.[21]

Many industrial enzymes, organic acids, amino acids, biofuels can be produced using agro-industrial wastes, which will, in turn, solve the problem of environmental pollution. Lignocellulolytic enzymes are of great value in paper and leather industries, bio-ethanol production, bio-leaching and bio-pulping processes.[22] The current demand for these enzymes in industrial sectors is not properly met and their full potential in other fields is not realized because of their high cost of production, the costly media and the pretreatment processes. Therefore, the major cellulolytic enzyme producers in the market, *viz*. Du-Pont-Genencor, Dyadic and Novozymes, have extensively tried to upgrade cocktails for low enzyme costs. No single microbial species is known to date to secrete all required cellulolytic enzymes in a balanced ratio and high titers.[23] The demand for carbohydrases, like amylase, cellulase and hemicellulase, will continue to rise, as they are part of the biofuel production process, the industry which is expected to generate USD 950 million by 2024. The lead of such industries is promoted by Brazil, France and USA.[24] The objective of this study is to enhance the production of lignocellulolytic enzymes by *Delftia* sp. PP4_S3, using submerged state fermentation of biodigested slurry, and further the utilization of this enzyme enriched slurry for biogas production using paddy straw.

EXPERIMENTAL

Materials

Biodigested slurry, which was used as a substrate for enzyme production, was obtained from a working biogas plant in the Demonstration area of the Department of Renewable Energy and Engineering (Punjab Agricultural University, Ludhiana). All the chemicals used for chemical analysis, including media and solution preparation, were of analytical grade. They were purchased from Hi-Media, SRL, Sigma and S.D. Fine Chemicals Pvt. Ltd. The bacterial culture of *Delftia* sp. PP4_S3 (GenBank Accession number JF274923.1) was obtained from the Department of Microbiology, PAU, Ludhiana.

Lignocellulolytic enzyme profile of *Deftia* sp. PP4_S3

The research work was conducted in the Biogas Laboratory and the Demonstration area of the Department of Renewable Energy and Engineering, Punjab Agricultural University, Ludhiana.

The cell suspension was prepared by inoculating the colony of *Delftia* sp. in nutrient broth (beef extract 1 g/L, yeast extract 2 g/L, peptone 5 g/L, sodium chloride 5 g/L), and incubating for 24 hours at 37± °C. The concentration of 10^7 cells/mL was used as cell suspension. 100 mL of nutrient broth, paddy straw broth (paddy straw chopped powder 10 g/L, dextrose 5 g/L, distilled water 1 L) and pasteurized 50% v/v biodigested slurry with water were dispensed separately in 250 mL Erlenmeyer flasks. The nutrient broth and paddy straw broth were autoclaved at 121 psi for 15 minutes, whereas unautoclaved biodigested slurry was used. The flasks were inoculated with 1% inoculum prepared by inoculating 100 mL of media with 1 mL well grown 24-h old culture of *Delftia* (10^7 cells/mL) in the respective media and incubated at 37±°C for 1-5 days. The supernatant was obtained by centrifugation at 4 °C, 10,000 rpm for 15 minutes, and analyzed for enzyme activities.

The experiment was performed in triplicate. The enzyme activities (U mL^{-1} of sample), such as exoglucanases, β-glucosidases, manganese peroxidase and lignin peroxidase, were measured in triplicates. The protein content (mg/mL) of the sample was analyzed by the method described by Lowry *et al*.,[25] using a UV-vis spectrophotometer (model 2800).

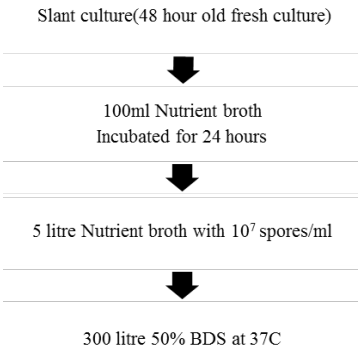

Figure 1: Inoculum preparation of *Delftia* sp.

Enzyme assay procedures

Exoglucanase (EC.3.2.1.91) activity was measured as described by the method by Mandels.[26] 1 mL of citrate buffer and Whatman filter paper strip (6 cm × 1 cm) were added into test tubes containing 0.5 mL supernatant, and incubated at 50 °C for one hour in a water bath. Controls devoid of filter paper strip were run simultaneously. The β-glucosidase (EC.3.2.1.21) activity was performed by the methods of Toyama and Ogawa.[27] In triplicate test tubes, containing 0.5 mL of enzyme extract, 0.5 mL of cellobiose solution (1%) was mixed in. Controls were also run simultaneously, devoid of cellobiose solution. The test tubes were incubated at 50 °C for 10 minutes in a water bath. Reducing sugars produced during the reaction were estimated using the DNS method by Miller.[28] The % light absorbance was recorded at 575 nm in a Hitachi UV-vis spectrophotometer (model 2800). The corresponding enzyme activity was read from the standard curve of reducing sugars, which was prepared by taking 50 to 300 µg/mL of glucose with ascending 50 µg intervals. The international unit of cellulases may be defined as 1 micromole of reducing sugar released per minute per milliliter of enzyme extract, measured as glucose:

$$\text{Reducing sugar µ mole/mL/min} = \frac{\text{mg of reducing sugar produced/mL}}{0.18 \times \text{Incubation period (min)}} \quad (1)$$

Manganese peroxidase (EC.1.11.1.13) activity was determined by the method of Paszczynski *et al.*[29] Guaiacol solution (3 mL), enzyme extract (0.2 mL), $MnSO_4$ (0.2 mL) and H_2O_2 (0.2 mL) were added to a cuvette and mixed. Changes in light absorbance were recorded for every 15 seconds up to 180 seconds at 465 nm against a blank without H_2O_2. The increase in O.D. by 0.001 in 60 seconds was taken as 1 unit. Lignin peroxidase (EC.1.11.1.14) (LiP) assay involved one mL of 10 mM veratryl alcohol, 1.5 mL of phosphate-citrate buffer, 0.4 mL of the enzyme extract and 0.1 mL of H_2O_2, which were mixed in a cuvette, and changes in light absorbance were recorded for every 15 seconds up to 180 seconds at 310 nm against a blank without H_2O_2. The extinction coefficient taken for MnP assay was $\acute{\varepsilon}_{465} = 12100\ M^{-1}\ cm^{-1}$ and for LiP assay it was $\acute{\varepsilon}_{310} = 9300\ M^{-1}\ cm^{-1}$. The enzyme activity is given as:

$$U/L = \frac{\Delta A \times V_t}{\acute{\varepsilon} \times V_e \times L} \times 10^6 \quad (2)$$

where $\Delta A = \frac{\text{Highest absorbance} - \text{Lowest absorbance}}{3\ \text{minutes}}$;

V_t = total volume in cuvette, V_e = volume of enzyme added, L = path length in cuvette.

The extracellular protein content of the enzyme extract was estimated according to the method given by Lowry *et al.*[25] Triplicate test tubes containing 1 mL of enzyme extract and 5 mL Reagent C were incubated at room temperature for 20 minutes. Then, 0.5 mL Reagent D was added and incubated for 10 minutes at room temperature. A control was run simultaneously replacing the enzyme with water. The light absorbance was measured at 520 nm using the Hitachi 2800 UV-vis spectrophotometer. The standard curve was prepared using a standard solution of Bovine Serum Albumin (BSA) in the concentration range of 0.1 to 1 mg/mL.

Multi-factorial optimization using response surface methodology

The experiment was performed in 17 sets, where 100 mL of biodigested slurry having concentrations 25% v/v, 50% v/v and 75% v/v were dispensed in Erlenmeyer flasks (250 mL capacity). The concentrations were formulated as mentioned in Kaur and Phutela.[5] Further, the slurries were inoculated with 1%, 2% and 3% culture (preparations described above) and incubated at 37± 2 °C. The readings were taken on the 4^{th}, 5^{th}, and 6^{th} day of incubation. The crude enzyme was extracted by centrifugation at 10,000 rpm for 15 minutes at 4 °C and the supernatant was analyzed for enzyme activities.

Submerged state fermentation for lignocellulolytic enzyme production

After optimization by response surface methodology (RSM) of lignocellulolytic enzyme production at laboratory scale, the submerged state fermentation studies have been conducted with 300 litres of biodigested slurry at 50% v/v concentration. The temperature of the biodigested slurry was maintained at 70 °C for 2 hours to pasteurise in the 500 litre capacity stirring batch fermentor (3 feet x 5 feet with tripod stand, 1.5 ft) with 1 hp speed control gear motor, 5 feet long bar with 4 blades and thermocouple to measure the inner temperature. It had an electrical panel with a display screen to show variation of temperature. The temperature was lowered down to 37 °C for inoculation of *Delftia* sp. culture.

The colony from 48 hour old *Delftia* culture was inoculated in 100 mL nutrient broth in 250 mL Erlenmeyer flask and incubated for 24 hours at 37 °C. 50 mL of this inoculum was transferred to 5 litre nutrient broth in Erlenmeyer flasks, and incubated for 24 hours at 37 °C. Inoculum preparation required two days. This 5 litre culture inoculum was transferred to 300 litre pasteurized 50% v/v biodigested slurry at 37 °C. Once the inoculum was prepared, the biodigested slurry already fed into the batch fermentor was inoculated with the bacterial culture at 37±2 °C temperature, 50% v/v biodigested slurry concentration, 1% inoculum, 7.0 pH, 25 rpm and 8 days of incubation period.

Supplementation of enzyme enriched biodigested slurry in paddy straw based biogas plant

The enzyme produced from the biodigested slurry in the batch fermentor was mixed with chopped paddy straw (300 kg), and was used for the biogas production trial in a mild steel biogas plant (400 Kg capacity) in the demonstration area of the department. To the enzyme activated biodigested slurry (150 litres), 30 kg of cattle dung and 3000 litres of water were also added in the biogas plant filled with chopped straw. Biogas produced at room temperature was measured by a digital meter connected at the top of the plant with the gas outlet. Data were collected until the biogas production reached the minimum level. A parallel control trial was also conducted, where 150 litre enzyme enriched slurry was replaced with 150 litre of biodigested slurry.

Proximate and chemical analyses of biodigested slurry

The biodigested slurry was analyzed for proximate (total solids, volatile solids and total organic carbon) and chemical composition (cellulose, hemicelluloses, lignin and silica) by standard methods of AOAC.[30]

RESULTS AND DISCUSSION

The present study was carried out to enhance lignocellulolytic enzyme production using biodigested slurry as a substrate. In this study, *Delftia* sp. PP4_S3, a bacterial culture was screened for lignocellulolytic enzyme production. The optimized technology for enzyme production at lab scale was upscaled to a large-scale fermentor of 500 litre capacity. Subsequently, it was supplemented with chopped paddy straw for enhancing biogas production. Several experiments were conducted, finally leading to increased biogas production from paddy straw using enzyme enriched biodigested slurry.

Enzymatic and biochemical analysis of biodigested slurry

The biodigested slurry (50% slurry) was analyzed for its enzyme activities, *viz*. exoglucanases, β-glucosidases, manganese peroxidase and lignin peroxidase. The biochemical composition, *i.e.* protein content and total reducing sugars, was also analyzed. The results from Table 1 indicate that the biodigested slurry is a good source of lignocellulolytic enzymes. Among all enzymes, β-glucosidases activity was the highest (3.89 U/mL), followed by exoglucanases (0.20 U/mL), MnP (0.036 U/mL) and LiP (0.029 U/mL) activities. The activity of lignin peroxidase was calculated as the lowest, *i.e.* 0.029 U/mL. The protein content of the digested slurry came out to be 2.76 mg/mL. This suggested that the biodigested slurry is a good, cheap substrate for lignocellulolytic enzyme production.

Kaur and Phutela[5] reported 85.0 U/mL of laccase activity, 0.05 U/mL of xylanase activity and protein content of 7.28 mg/mL in the biodigested slurry. The variation in the BDS depends on the cattle feed and the physiochemical conditions of operating feedstock. Another study reported the isolation of bacteria from soil and the valorization of lignocellulosic residues for cellulase enzyme production by submerged state fermentation. As a result, *Pseudomonas stutzeri* gave the highest cellulase activity of 170.9±4.1 (IU/mL/min), followed by *Bacillus paralichniformis*, *Bacillus wiedmanni* in eucalyptus leaves containing media at 37 °C for 24 h incubation period.[31]

Lignocellulolytic enzyme profile and biochemical constituents

Table 2 shows the lignocellulolytic enzyme profile of *Delftia* sp. in 50% v/v slurry concentration, 1% inoculum incubated for 5 days in the incubator. The results indicate exoglucanase activity of 0.036 U mL^{-1}, β-glucosidase activity of 1.459 U mL^{-1}, MnP

activity of 0.244 U mL^{-1} and LiP activity of 0.111 U mL^{-1}. The biochemical constituents observed reached 2.47 mg/mL and the protein content – 0.511 mg/mL. Thus, *Delftia* produced appreciable enzyme activities and has potential for further scale-up processes. Kamsani *et al*.[32] studied the production of lignocellulolytic enzymes by microbes isolated from *Bulbitermes* sp. termite gut in SSF. They reported maximum lignin peroxidase (729.12 U/g) and β-glucosidase activity (22.97 U/g) in *Aspergillus* sp., while maximum endoglucanase (138.77 U/g) and manganese peroxidase activity (47.73 U/g) in *Bacillus* sp. B1. The highest activities of exoglucanase (32.16 U/g) and laccase (71.18 U/g) were reported in *Bacillus* sp. B2. Zhang *et al.* studied enzymatic hydrolysis of lignin by lignolytic enzymes and analyzed chemical bond cleavage, benzene ring opening and groups in alkali lignin, and concluded that the optimum lignin degradation – of 28.98% – occurred effectively by the combination of Lac, LiP and MnP.[33]

Table 1
Enzyme activities and biochemical constituents of biodigested slurry

S. No.	Enzyme activities and biochemical constituents	Values
1	Exoglucanases (U/mL)	0.20
2	B-glucosidases (U/mL)	3.89
3	Manganese peroxidises (U/mL)	0.036
4	Lignin peroxidises (U/mL)	0.029
5	Reducing sugars (mg/mL)	4.26
6	Protein (mg/mL)	2.76

Table 2
Lignocellulolytic enzyme profile and biochemical constituents

Lignocellulolytic enzymes	Enzyme activities
Exoglucanases (U mL^{-1})	0.036±0.006
β-glucosidases (U mL^{-1})	1.459±0.045
MnP (U mL^{-1})	0.244±0.025
LiP (U mL^{-1})	0.111±0.009
Reducing sugars (mg/mL)	2.47±0.011
Protein content (mg/mL)	0.511±0.044

Culture conditions: incubation temperature 37±2 °C, incubation period: 5 days, inoculation: 1% inoculum, biodigested slurry concentration 50%. Data represent the average of triplicate trials; ±values indicate standard error

Statistical optimization of enzyme production by *Delftia sp* from digested biogas slurry by RSM

The effect of three factors, *viz.* slurry concentration, spore concentration and incubation period, were studied for *Delftia* sp. using Multilevel Factorial Design, as shown in Table 3. The independent factors included spore concentration (10^6, 10^7, 10^8 spores/mL), slurry concentration (25%, 50% and 75%) and incubation period (4, 5 and 6 days). The various response variables were exoglucanases (U mL^{-1}), β-glucosidases (U mL^{-1}), manganese peroxidases (UL^{-1} of BDS), lignin peroxidases (UL^{-1} of BDS) and protein (mg mL^{-1}). It was observed that maximum exoglucanase activity (0.98U mL^{-1}) was obtained at 50% slurry concentration; 1% inoculum for 5 days, while second highest activity (0.92 U mL^{-1}) was close to the highest activity at 50% slurry concentration, 3% inoculum for 5 days. The maximum activity for β-glucosidases (1.97 U mL^{-1}) was observed at 50% slurry concentration, 1% inoculum for 5 days, followed by 1.91 U mL^{-1} activity at 50% slurry concentration, 3% inoculum for 5 days. Mnp activity (17.86 U L^{-1}) was observed to be maximum at 50% slurry concentration, 3% inoculum for 5 days, followed by 15.42U L^{-1} at 50% slurry concentration, 1% inoculum for 5 days. The highest LiP activity of 93.87 U L^{-1} was observed at 50% slurry concentration, 1% inoculum for 5 days, followed by 88.64 U L^{-1} at

75% slurry concentration, 1% inoculum for 4 days.

The F-value 4.58 of manganese peroxidase suggested that the model was significant. Linear term (B) and quadratic terms (B^2, C^2) were statistically significant, with p values <0.05. It was observed that the inoculum (0.0024) was the most significant factor affecting the enzyme production, followed by slurry concentration (0.1937) and incubation period (0.5386).

Table 4 tabulates the ANOVA data, partitioning the variability in MnP into separate pieces for each of the effects. It then tests the statistical significance of each effect by comparing the mean square against an estimate of the experimental error. In this case, 4 effects have P values less than 0.05, indicating that they are significantly different from zero at 95% confidence level. The R^2 statistic indicates that the model as fitted explains 80.46% of the variability in MnP. The adjusted R^2 statistic, which is more suitable for comparing models with different number of independent variables, is 62.48%.

Figure 2 presents the relationship between the predicted value (Y) and the actual value (X). The straight line verified the normality of data. Since the majority of the points were close to the line, this indicates that the response from the model was in agreement with the actual values of independent variables. The high degree of agreement showed that CCD can successfully be utilized as a reliable tool for optimization and supported the hypothesis that the model was sufficient to explain the response of the experimental observations related to manganese peroxidase production.

Table 4
ANOVA for quadratic model: MnP

Source	Sum of squares	Df	Mean square	F-value	p-value	
Model	76.79	9	8.53	4.58	0.0131	significant
A-incubation period	0.7559	1	0.7559	0.4053	0.5386	
B-inoculum	30.12	1	30.12	16.15	0.0024	
C-slurry concentration	3.62	1	3.62	1.94	0.1937	
AB	0.3300	1	0.3300	0.1770	0.6829	
AC	5.81	1	5.81	3.12	0.1079	
BC	3.99	1	3.99	2.14	0.1744	
A^2	2.26	1	2.26	1.21	0.2966	
B^2	10.74	1	10.74	5.76	0.0373	
C^2	16.32	1	16.32	8.75	0.0143	
Residual	18.65	10	1.86			
Lack of fit	13.23	7	1.89	1.05	0.5350	not significant
Pure error	5.41	3	1.80			
Cor. total	95.44	19				

R-squared = 80.46 percent; R-squared (adjusted for d.f.) = 62.48 percent

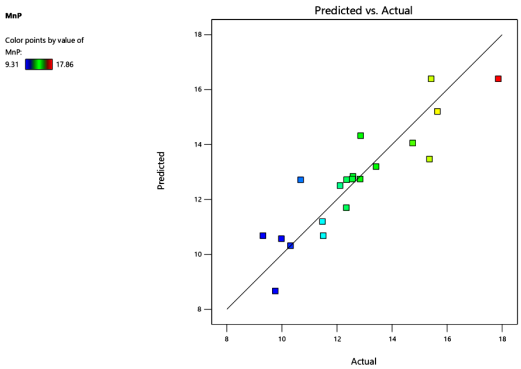

Figure 2: Predicted *vs* actual values of manganese peroxidase

Table 3
Statistical optimization of enzyme production by *Delftia* sp. from digested biogas slurry by RSM

Set no.	Incubation period (days)	Inoculum (%)	Slurry concentration (%)	Exoglucanases (U mL^{-1} BDS)	β-Glucosidases (U mL^{-1} BDS)	Manganese peroxidase (UL^{-1} BDS)	Lignin peroxidase (UL^{-1} BDS)	Protein (mg mL^{-1})
1	5	3	75	0.85	1.85	12.86	85.48	2.56
2	4	3	25	0.56	1.65	15.65	80.76	2.21
3	4	3	75	0.86	1.87	13.42	79.68	2.54
4	6	2	75	0.79	1.79	12.34	83.4	1.81
5	6	1	50	0.64	1.57	10.68	83.9	1.18
6	4	1	50	0.74	1.81	12.58	83.21	1.46
7	6	3	75	0.89	1.91	14.75	81.34	1.97
8	5	1	25	0.46	1.34	10.31	81.49	0.85
9	4	1	25	0.51	1.48	9.98	83.58	1.09
10	5	1	75	0.62	1.85	12.35	78.89	1.63
11	5	2	25	0.68	1.82	11.5	64.98	0.78
12	6	3	25	0.84	1.87	12.11	87.61	1.14
13	5	1	50	0.98	1.97	15.42	93.87	1.96
14	4	1	75	0.92	1.79	11.47	88.64	2.4
15	6	1	25	0.68	1.15	9.76	77.32	0.75
16	4	2	50	0.91	1.88	12.84	86.66	2.54
17	5	3	50	0.92	1.91	17.86	85.48	3.28

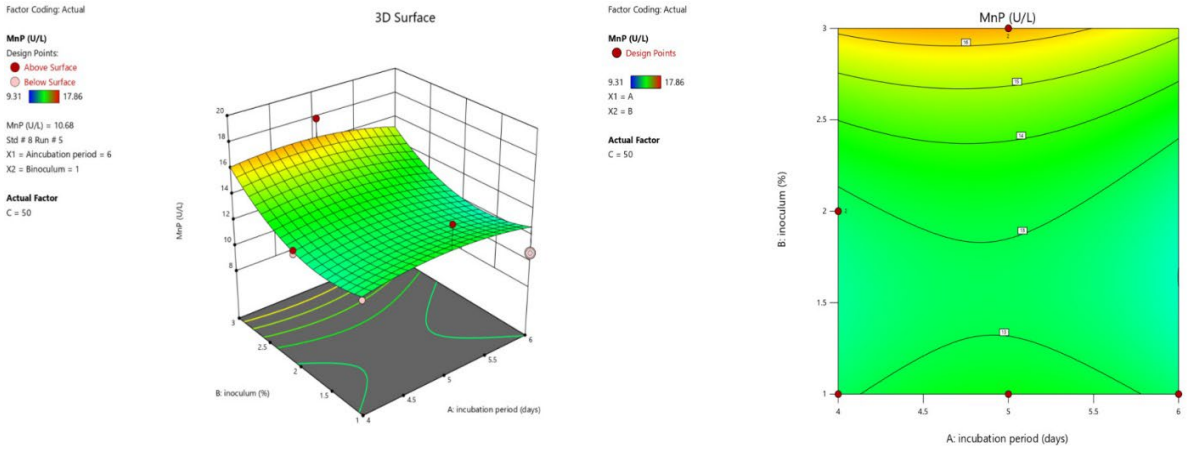

Figure 3: Surface plot (left) and contour plot (right) of manganese peroxidase showing the effect of interaction between inoculum and incubation period

Figure 3 shows the comparative effects of any two independent variables on enzyme production in the form of surface plots (3D) and contour plots (2D), while keeping other variables at their central point values. These graphs are representations of the regression equation for optimizing the reaction conditions.

Surface and contour plots indicated that the interaction between inoculum and incubation period had an appreciable effect on MnP production, with the highest activity observed at 2.5-3% and incubation period of 4 to 5.5 days. Also, low inoculum had a significant effect on enzyme production and enzyme activity decreased with a decrease in inoculum concentration. The optimum inoculum concentration observed was 3% from 4 to 6 days of incubation.

The F-value 7.15 of lignin peroxidase suggested that the model fitted into the quadratic polynomial equation. Linear terms (B, C), interacting terms (AB, AC) and quadratic terms (C^2) were statistically significant with p values <0.05. It was observed that inoculum (0.0128) was the most significant factor affecting the enzyme production, followed by slurry concentration (0.0450) and incubation period (0.086).

Table 5 shows the ANOVA data, partitioning the variability in LiP into separate pieces for each of the effects. It then tests the statistical significance of each effect by comparing the mean square against an estimate of the experimental error.

Table 5
ANOVA for quadratic model: Lip

Source	Sum of squares	Df	Mean square	F-value	p-value	
Model	613.82	9	68.20	7.15	0.0025	significant
A-incubation period	34.45	1	34.45	3.61	0.0866	
B-inoculum	87.29	1	87.29	9.15	0.0128	
C-slurry concentration	50.08	1	50.08	5.25	0.0450	
AB	188.05	1	188.05	19.70	0.0013	
AC	195.23	1	195.23	20.45	0.0011	
BC	42.04	1	42.04	4.40	0.0622	
A^2	0.9072	1	0.9072	0.0950	0.7642	
B^2	3.23	1	3.23	0.3383	0.5737	
C^2	88.97	1	88.97	9.32	0.0122	
Residual	95.45	10	9.55			
Lack of fit	77.35	7	11.05	1.83	0.3338	not significant
Pure error	18.11	3	6.04			
Cor. total	709.27	19				

R-squared = 86.54 percent; R-squared (adjusted for d.f.) = 74.43 percent

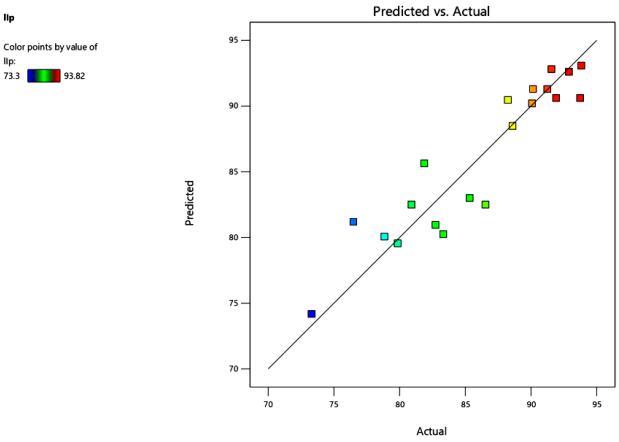

Figure 4: Predicted *vs* actual values of lignin peroxidase

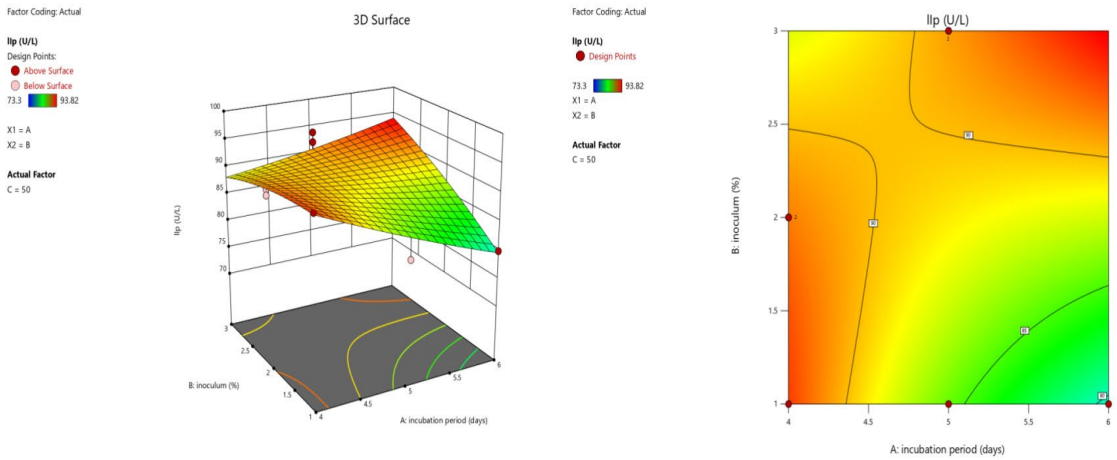

Figure 5: Surface plot (left) and contour plot (right) of lignin peroxidase showing the effect of interaction between inoculum % and incubation period

In this case, 6 effects have P values less than 0.05, indicating that they are significantly different from zero at 95% confidence level. The R^2 statistic indicates that the model as fitted explains 86.54% of the variability in LiP. The adjusted R^2 statistic, more suitable for comparing models with different number of independent variables, is 74.43%.

Figure 4 presents the relationship between the predicted value (Y) and actual value (X). The straight line verified the normality of data, which indicates that the response from the model was in agreement with actual values of independent variables. Figure 5 shows the comparative effects of two independent variables, *i.e.* inoculum concentration and incubation period, on enzyme production in the form of surface plots (3D) and contour plots (2D), while keeping other variables at their central point values. These graphs are representations of the regression equation for optimizing the reaction conditions. Surface and contour plots indicate that the interaction between inoculum and incubation period had a strong effect on LiP production, with the highest activity observed at 1-2% and 2.5-3% in 5-6 days. Also, incubation period had a significant effect on enzyme production, and enzyme activity increased with an increase in the number of days and with increasing inoculum concentration. The optimum inoculum range observed was 1-2% and incubation period was 5-6 days.

The large F-value (10.05) of β-glucosidase indicated that the model fitted into the quadratic polynomial equation. Linear terms (B, C), interacting terms (AB, BC) and quadratic terms (C^2) were statistically significant with p values <0.05. It was observed that inoculum (0.0004) was the most significant factor affecting the

enzyme production, followed by slurry concentration (0.0008) and incubation period (0.3328).

Table 6 shows the ANOVA analysis, partitioning the variability in β-glucosidase into separate pieces for each of the effects. It then tests the statistical significance of each effect by comparing the mean square against an estimate of the experimental error. In this case, 6 effects have P values less than 0.05, indicating that they are significantly different from zero at 95% confidence level. The R^2 statistic indicates that the model as fitted explains 90.05% of the variability in β-glucosidase. The adjusted R^2 statistic is 81.09%. Figure 6 presents the relationship between the predicted value (Y) and actual value (X). The straight line verified the normality of data, supporting the hypothesis that the model was sufficient to explain the response of the experimental observations related to β-glucosidase production. Figure 7 shows the comparative effects of two independent variables, *i.e.* inoculum concentration and incubation period, on enzyme production in the form of surface plots (3D) and contour plots (2D). The plots indicate that the interaction between inoculum and incubation period had a strong effect on enzyme production, with the highest activity observed at 2.5-3% in 4-6 days. Also, low inoculum had a significant effect on enzyme production and enzyme activity decreased with a decrease in inoculum. The optimum inoculum range observed was 2-5% and optimum incubation period – of 4-6 days.

Table 6
ANOVA for quadratic model: β-glucosidase

Source	Sum of squares	df	Mean square	F-value	p-value	
Model	0.7915	9	0.0879	10.05	0.0006	significant
A-incubation period	0.0091	1	0.0091	1.04	0.3328	
B-inoculum	0.2385	1	0.2385	27.26	0.0004	
C-slurry concentration	0.1979	1	0.1979	22.62	0.0008	
AB	0.0742	1	0.0742	8.48	0.0155	
AC	0.0002	1	0.0002	0.0249	0.8777	
BC	0.0612	1	0.0612	7.00	0.0245	
A^2	0.0115	1	0.0115	1.31	0.2786	
B^2	0.0090	1	0.0090	1.03	0.3350	
C^2	0.0691	1	0.0691	7.90	0.0184	
Residual	0.0875	10	0.0087			
Lack of fit	0.0368	7	0.0053	0.3118	0.9076	not significant
Pure error	0.0507	3	0.0169			
Cor. total	0.8790	19				

R-squared = 90.05 percent; R-squared (adjusted for d.f.) = 81.09 percent

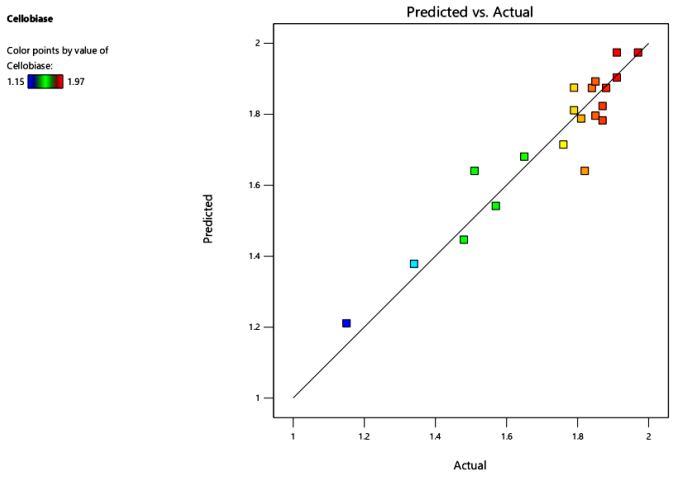

Figure 6: Predicted *vs* actual values of β-glucosidase

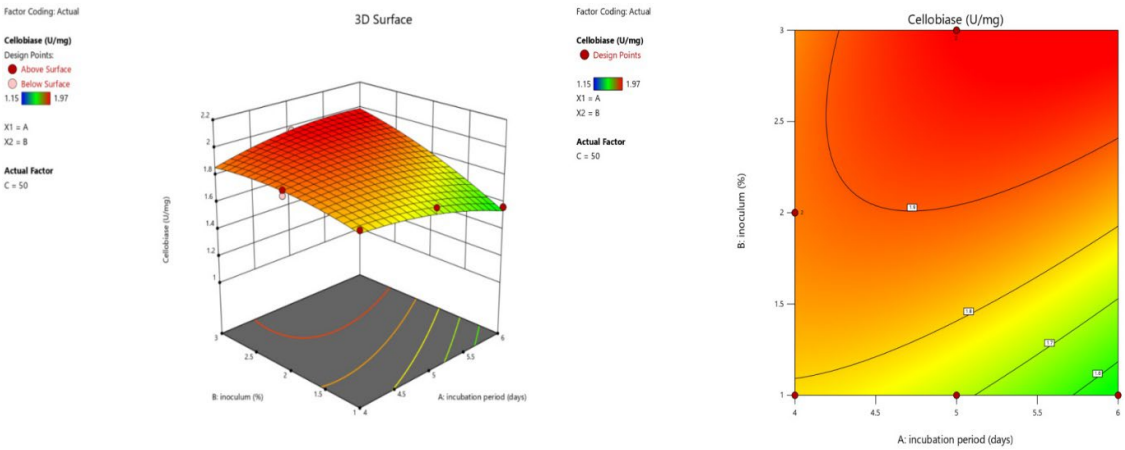

Figure 7: Surface plot (left) and contour plot (right) of β-glucosidase showing the effect of interaction between inoculum and incubation period

The F-value (6.93) of exoglucanases indicated that the model fitted into the quadratic polynomial equation. Linear terms (B, C), interacting terms (AB, AC) and quadratic terms (C^2) were statistically significant with p values <0.05. It was observed that inoculum (0.0008) was the most significant factor affecting the enzyme production, followed by slurry concentration (0.0058) and incubation period (0.5410). Table 7 tabulates the ANOVA data and partitions the variability in exoglucanases into separate pieces for each of the effects. It then tests the statistical significance of each effect by comparing the mean square against an estimate of the experimental error. In this case, 6 effects have P values less than 0.05, indicating that they are significantly different from zero at 95% confidence level. The R^2 statistic indicates that the model as fitted explains 86.18% of the variability in exoglucanases, and the adjusted R^2 is 73.74%.

Figure 8 presents the relationship between the predicted value (Y) and actual value (X). The straight line verified the normality of data. Exoglucanase activity observed at both ends was 0.7 U/mg and 0.9 U/mg, respectively, while it was restricted between 0.7 U/mg and 0.9 U/mg in the case of inoculum and incubation period. Surface and contour plots (Fig. 9) indicate that the interaction between inoculum and incubation period had a strong effect on exoglucanases production, with the highest activity observed at 2-3% inoculum and incubation period of 5.5 to 6 days. Also, negligible activity was observed on initial days and low inoculum concentration.

The F-value (19110.11) of protein suggested that the model was significant. Linear terms (A, B, C), interacting terms (AB, AC, BC), and quadratic terms (A^2, B^2, C^2) were statistically significant with p values <0.05. It was observed that inoculum, slurry concentration and incubation period (<0.0001) were significant factors affecting the enzyme production.

Table 8 indicates the ANOVA data and partitions the variability in protein into separate pieces for each of the effects. It then tests the statistical significance of each effect by comparing the mean square against an estimate of the experimental error. In this case, 10 effects have P values less than 0.05, indicating that they are significantly different from zero at 95% confidence level. The R^2 statistic indicates that the model as fitted explains 99.9% of the variability in protein; the adjusted R^2 is 99.9%. Figure 10 presents the relationship between the predicted value (Y) and actual value (X). The straight line verified the normality of data. This was followed by inoculum concentration, where the change in activity was less significant. Incubation period also showed a curvilinear response from the central point, indicating its significant effect on protein production. Surface and contour plots (Fig. 11) indicated that the interaction between inoculum and incubation period had a strong effect on protein production. The protein value decreases with an increase in incubation period from 4 to 6 days. Also, as inoculum concentration is decreased, there is a curvilinear decrease in protein.

Table 7
ANOVA for quadratic model: exoglucanase

Source	Sum of squares	df	Mean square	F-value	p-value	
Model	0.3617	9	0.0402	6.93	0.0028	significant
A-incubation period	0.0023	1	0.0023	0.4006	0.5410	
B-inoculum	0.1296	1	0.1296	22.34	0.0008	
C-slurry concentration	0.0706	1	0.0706	12.17	0.0058	
AB	0.0351	1	0.0351	6.06	0.0336	
AC	0.0745	1	0.0745	12.85	0.0050	
BC	0.0004	1	0.0004	0.0735	0.7919	
A^2	0.0075	1	0.0075	1.29	0.2831	
B^2	0.0267	1	0.0267	4.61	0.0574	
C^2	0.0350	1	0.0350	6.04	0.0338	
Residual	0.0580	10	0.0058			
Lack of fit	0.0330	7	0.0047	0.5638	0.7605	not significant
Pure error	0.0250	3	0.0083			
Cor. total	0.4197	19				

R-squared = 86.18 percent; R-squared (adjusted for d.f.) = 73.74 percent

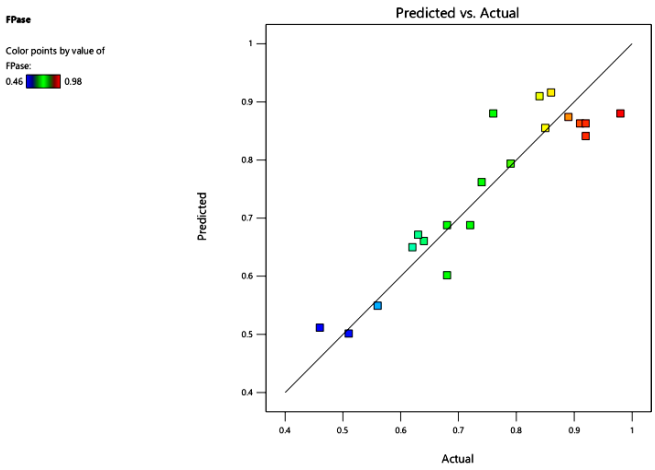

Figure 8: Predicted *vs* actual values of exoglucanases

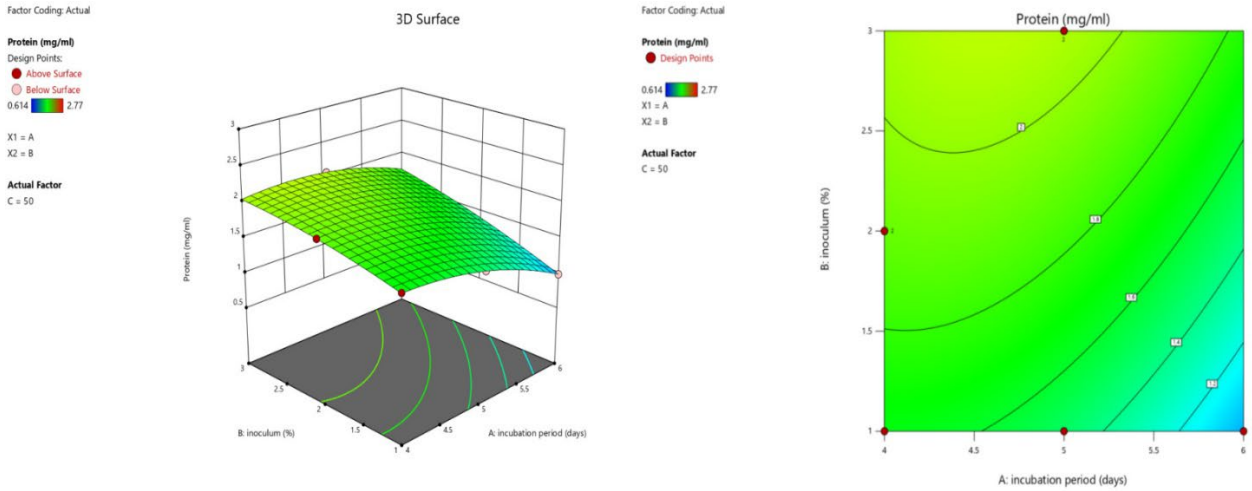

Figure 9: Surface plot (Left) and Contour plot (Right) of exoglucanase showing the effect of interaction between inoculum and incubation period

Table 8
ANOVA for quadratic model: protein

Source	Sum of squares	df	Mean square	F-value	p-value	
Model	6.49	9	0.7213	19110.11	< 0.0001	significant
A-incubation period	0.6210	1	0.6210	16452.67	< 0.0001	
B-inoculum	1.20	1	1.20	31776.07	< 0.0001	
C-slurry concentration	3.66	1	3.66	96895.49	< 0.0001	
AB	0.0781	1	0.0781	2068.76	< 0.0001	
AC	0.0241	1	0.0241	637.90	< 0.0001	
BC	0.1174	1	0.1174	3109.71	< 0.0001	
A^2	0.1221	1	0.1221	3233.96	< 0.0001	
B^2	0.0136	1	0.0136	359.84	< 0.0001	
C^2	0.0002	1	0.0002	5.45	0.0418	
Residual	0.0004	10	0.0000			
Lack of fit	0.0003	7	0.0000	2.81	0.2135	not significant
Pure error	0.0001	3	0.0000			
Cor. total	6.49	19				

R-squared = 99.9 percent; R-squared (adjusted for d.f.) = 99.9 percent

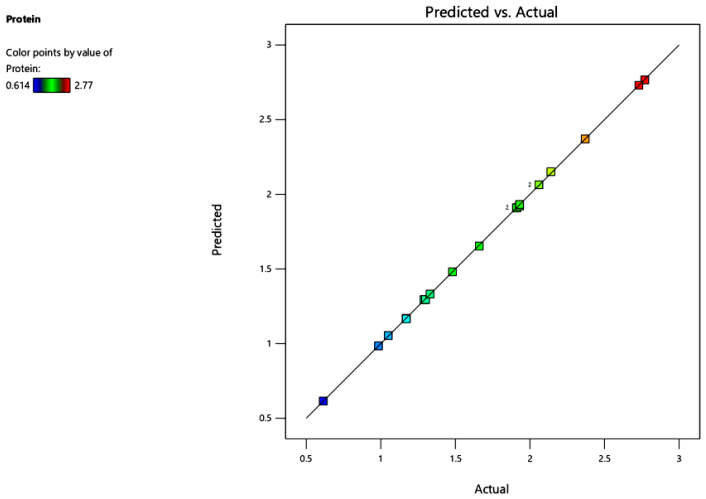

Figure 10: Predicted *vs* actual values of protein

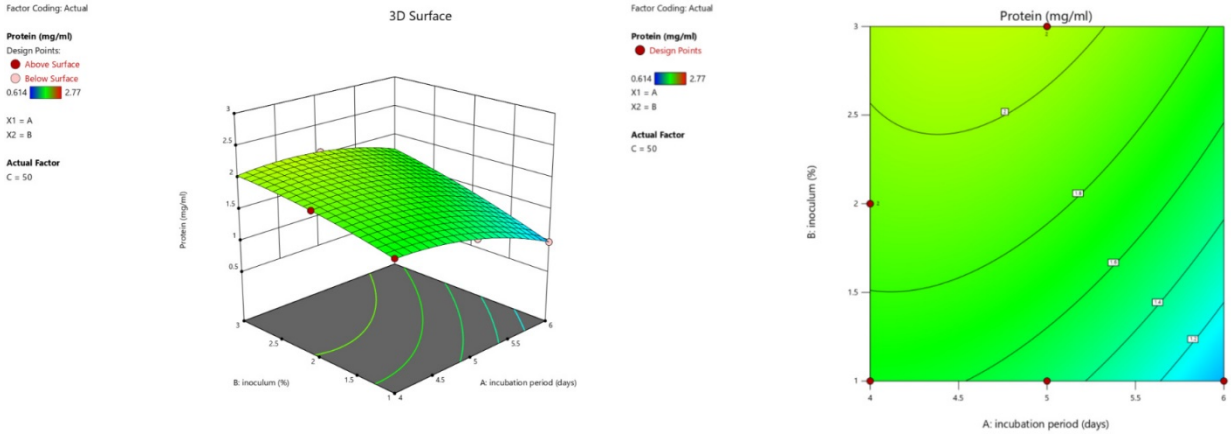

Figure 11: Surface plot (left) and contour plot (right) of protein showing the effect of interaction between inoculum and incubation period

Enzyme activities in different conditions

Table 9 shows the enzyme activities observed under three conditions, including unoptimized, optimization by one factor at a time and optimization by RSM. The exoglucanase activity in *Delftia* showed a significant increase in enzyme

activity against unoptimized conditions – a 27 fold increase. However, the increase in β-glucosidase activity was found to be only 1.35 fold in *Delftia*. Ahmad *et al*.[34] reported that RSM for cellulase production from *Aneurinibacillus aneurinilyticus* isolated from Himalayan freshwater showed a 60 fold increase in enzyme activities, compared to unoptimized culture medium. Hence, RSM leads to increases in enzyme yields under specific cultural conditions.

Comparison of enzyme activities of *Delftia* sp. in different media at lab scale

Various enzyme activities, like exoglucanases, β-glucosidases, manganese peroxidase and lignin peroxidase, were studied for *Delftia* sp. using nutrient broth, potato dextrose broth and 50% biodigested slurry. Table 10 shows that maximum enzyme activities were observed mainly in 50% biodigested slurry, including exoglucanases (0.035U mL^{-1}), β-glucosidase (1.391 U mL^{-1}), manganese peroxidase (0.077U mL^{-1}) and lignin peroxidase (0.116U mL^{-1}), followed by paddy straw broth and then nutrient broth. The highest enzyme activities in the 50% slurry may be attributed to the stability, nutrient availability and specificity of the substrate, allowing enzymes to be active for 5 days of incubation period. However, this trend was not observed in the case of LiP activity, which was maximum in paddy straw broth (0.197 U mL^{-1}), followed by nutrient broth (0.124 U mL^{-1}) and 50% biodigested slurry (0.116 U mL^{-1}). The protein content was observed to be maximum in its usual nutrient broth, but most of enzymes were maximum in the slurry medium. Kaur and Phutela[5] compared lignocellulolytic enzyme activities in nutrient broth and paddy straw based nutrient broth and reported maximum exoglucanases of 1.36 U mL^{-1}, β-glucosidases of 1.33 U mL^{-1} when incubated with 1% inoculum at 37 °C, pH 7, in paddy straw based nutrient broth.

Table 9
Enzyme activities of *Delftia* sp. under different conditions

Conditions	Exoglucanase (U mL^{-1})	Fold increase	β-glucosidase (U mL^{-1})	Fold increase
Unoptimized	0.036	1	1.45	1
Optimization by RSM	0.980	27.23	1.97	1.35

Table 10
Comparison of enzyme activities of *Delftia* sp. in different media

Enzyme activities (*Delftia*)	Different media used		
	Nutrient broth	Paddy straw broth	50% BDS slurry
Exoglucanases (U mL^{-1})	0.019±0.003	0.028±0.004	0.035±0.002
β-glucosidases (U mL^{-1})	1.036±0.001	1.282±0.001	1.391±0.010
Manganese peroxidase (U mL^{-1})	0.073±0.033	0.012±0.050	0.077±0.020
Lignin peroxidase (U mL^{-1})	0.124±0.017	0.197±0.001	0.116±0.006
Protein content (mg mL^{-1})	0.665±0.031	0.607±0.010	0.481±0.026
CD (5%)	0.574	0.486	0.477

Culture conditions: incubation temperature: 37±2 °C, incubation period: 5 days, inoculum 1%, pH 7
Data represent average of triplicates; ±values indicate standard error

Table 11
Submerged state fermentation for lignocellulolytic enzyme production

Incubation period (days)	Enzyme activities				
	Exoglucanases (U mL^{-1})	β-glucosidase (U mL^{-1})	MnP (U mL^{-1})	LiP (U mL^{-1})	Protein (mg mL^{-1})
0	0.006±0.005	1.01±0.027	0.023±0.001	0.001±0.001	0.215±0.002
3	0.014±0.002	1.15±0.015	0.056±0.005	0.042±0.002	0.359±0.015
4	0.028±0.002	1.105±0.002	0.092±0.001	0.060±0.001	0.499±0.002
5	0.0115±0.002	0.293±0.008	0.015±0.001	0.050±0.002	0.426±0.011
CD (5%)	0.169	0.142	0.285	0.215	0.322

Culture conditions: incubation temperature: 37±2 °C, incubation period 3-5 days, inoculum: 5 litres in 300 litre slurry (50%). Data represent average of triplicates; ±values indicate standard error

Submerged state fermentation for lignocellulolytic enzyme production by *Delftia* sp. using BDS as a substrate

Table 11 shows the lignocellulolytic enzyme production by *Delftia* sp. in a large scale fermentor (500 liter capacity), filled with 300 L BDS (50% diluted by water). The BDS was pasteurised at 70 °C for 2 hours, then temperature was lowered down to 37±2°C, and it was inoculated with 1% culture. Enzyme activities were taken for a period of 5 days. The enzyme production increased from day zero and reached the maximum on the 4th day, with exoglucanase activity of 0.028 U mL^{-1}, MnP activity of 0.092 U mL^{-1} and LiP activity of 0.060 U mL^{-1}. This was followed by a decrease in enzyme activities on the 5th day. The bacteria were able to retain their enzyme activity for 4 to 5 days as a result of the stable medium for enzyme production, which would otherwise be lost after 48 hours in a different medium. This may be due to slow kinetics in reaching the optimum phase. Vicuna[35] reported less prolific lignin degrading bacteria, namely *Cellulomonas*, *Pseudomonas* and *Clostridium thermocellum*, to set a gene pool for lignocellulose degradation to be used in lignocellulase engineering. Cunha *et al*.[36] reported that the selection of operational variables had a significant impact on cellulase enzyme production in stirred bioreactor fermentations using sugarcane bagasse. Smaller stirred particles provide uniformity in the bioreactor. Thus, bagasse chunks less than 0.5 mm in size showed maximum endoglucanase (1599 IU L^{-1}) and xylanase (4212 IU L^{-1}) at pH 5.0 and 700 rpm speed. Another study reported the effect of different agro-residues on lignocellulolytic enzyme production from fungus, *Bjerkandera adusta* BRFM 1916, native to the Algerian forest. Among different lignocellulosic residues, namely wheat bran, wheat straw, barley bran, orange peels and grape pulp, the highest CMCase (690±0.066 U L^{-1}) and β-glu (253 U L^{-1}) were reported in wheat bran and orange peel, respectively.[37]

Cost savings for lignocellulolytic enzyme units produced in fermentor

Table 12 shows the lignocellulolytic enzyme units produced by *Delftia* sp. per 300 L BDS per batch in a 500 L fermentor. The enzyme units obtained from 300 L of BDS were 8400 units of exoglucanase, 331500 units of β-glucosidase, 27600 units of manganese peroxidase and 18000 units of lignin peroxidase. The cost of nutrient broth for 100 L of medium is 4170 INR (by adding the cost of all the individual components: peptone, beef extract, NaCl). The cost for 300 L of nutrient broth would be 12510 INR. Hence, BDS can be an alternative for producing maximum enzyme units with minimal costs and saving up to Rs 12510 for 300 L of medium at large scale.

Effect of supplementation of enzyme enriched biodigested slurry with paddy straw on biogas production

Table 13 shows the biogas production of the paddy straw based biogas plant, supplemented with 100 L of enzyme enriched biodigested slurry of *Delftia* sp., which includes 300 kg of paddy straw, 30 kg cattle dung and 30 kg of 50% biodigested slurry. The total biogas production of 101.47 cubic meters was recorded for the period of 4 months – February to May. The biogas L/Kg PS, L/Kg TS PS and L/Kg VS PS were 338.234, 356.035 and 424.407, respectively. Zielinski *et al*.[38] reported that biogas yield increased by supplementation of 0.5% of the fresh biomass dose of cattle feed.

Table 12
Cost savings for lignocellulolytic enzymes using *Delftia* sp.

Enzyme activities	Enzyme units mL^{-1} BDS*	Enzyme units/ 300 L BDS	Savings for enzyme production
Exoglucanase	0.028	8400	Cost of nutrient broth** per
β-glucosidase	1.15	331500	100 L= 4170/-
Manganese peroxidase	0.092	27600	Cost of nutrient broth per
Lignin peroxidase	0.060	18000	300 L=12510/-

*Values taken from Table 4; **HiMedia components were used for nutrient broth

Li *et al*.[39] reported that biological pretreatment of corn straw with mixed microbes, including *Phaenarochaete chrysosporium*, *Coriolus versicolor*, *Trichodermaa viride*, *Aspergillus niger*, *Bacillus circulans* and *Pseudomonas aeroginosa*, increased the degradation of cellulose, hemicelluloses and lignin by 34%, 44% and 49%, respectively. The methane production with biological pretreatment reached 5721 mL, while the untreated yield was 2469 mL. A study reported enhanced biogas production by using *Lantana camara* as a substrate with cow dung as an inoculum. The inoculum was bioaugmented with cellulolytic bacteria namely, *Microbacterium* sp. (DSB1) and *Arthrobacter* sp. (DSB12) that produced 950 L/kg and 980 L/kg VS biogas, respectively.[40]

Table 13
Biogas production from paddy straw mixed with enzyme enriched biodigested slurry using *Delftia* sp.

Composition of feedstock	Total biogas (Litres)	Biogas (L/Kg PS)	Biogas (L/Kg TS PS)	Biogas (L/Kg VS PS)
PS = 300 kg, CD = 30 kg, BDS = 30 kg, 100 L lignocellulosic enzyme enriched BDS (50%) inoculated with *Delftia* sp.	101470 or (101.47 m^3)	338.234	356.035	424.407

Table 14 exhibits the monthly profile of biogas production. Maximum biogas production of paddy straw treated with *Delftia* sp. was observed in the month of April, *i.e.* 28.93% of total gas produced, followed by 26.71% in the month of May. Table 14 shows that biogas production increased with an increase in environment temperature initially and then there is a slight decrease in the production in the month of May (Fig. 12). The total biogas produced in four months was 101.47 m^3, which was higher than that produced by the control (65 m^3) by 35.94%. As the experiment was started in the month of February, only 44.34% biogas was harvested within the first 2 months. This indicates that *Delftia* sp. was able to degrade paddy straw efficiently and thus increase biogas production.

The proximal analysis results of feedstock, before and after digestion, shown in Table 15 indicate the consumption of total solids (TS), volatile solids (VS) and organic carbon consumption. TS of the feedstock treated with *Delftia* sp. was reduced from 16.84% to 10.77%, resulting in a 6.07% decrease. Similarly, VS was reduced from 81.1% to 71.61%, with a 9.49% decrease. Total solids represents the ratio of organic material percentage without water to the total weight of the organic material. As known, biodegradation results in conversion of solid organic material into gaseous form, which results in overall loss of organic material.

Total solids consist of volatile solids and ash content. During anaerobic digestion, volatile solids are converted into gases, which results in a decrease in volatile solids in the bio-digester and, simultaneously, in an increase in ash percentage (but the ash content remains the same). The ash content increased by 8.13% in *Delftia* sp. As biogas is mainly composed of methane and carbon dioxide, total organic carbon also decreases in the whole process.

Table 14
Upscaled monthly biogas production from paddy straw supplemented with enzyme enriched (*Delftia* sp.) BDS

Month	Temperature (°C)	Control	Biogas production of paddy straw supplemented with enzyme enriched *Delftia* sp. BDS (in m^3)	Percentage of biogas in 4 months (%)
February	17.51	7.98	19.79	19.50
March	21.19	16.52	25.21	24.84
April	27.39	19.02	29.36	28.93
May	30.18	21.48	27.11	26.71
Total biogas production		**65 m^3**	**101.47 m^3**	

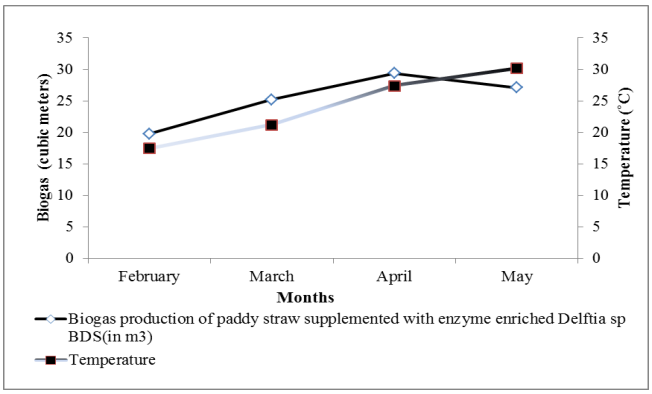

Figure 12: Variation of biogas production with temperature and time

Table 15
Proximate and chemical analyses of feedstock before and after digestion process for *Delftia* sp.

Proximate composition (%)			Chemical composition (%)			
Before digestion						
Total solids (TS)	Volatile solids (VS)	Ash	Cellulose	Hemicelluloses	Lignin	Silica
16.84	81.1	17.09	29.9	21.1	9.6	9.4
After digestion						
10.77	71.61	25.22	21.5	13.57	9.3	14.9
Percentage change						
6.07(↓)	9.49(↓)	8.13(↑)	8.4(↓)	7.53(↓)	0.3(↓)	5.5(↑)

A decrease in cellulose, hemicellulose and lignin content and an increase in silica percentage have been observed after biogas production. The percentage change in cellulose observed was 8.4% in *Delftia* sp. and that of hemicelluloses was 7.53%. Both cellulose and hemicelluloses are easily hydrolyzed due to their branched and amorphous structure, having short lateral chains and low molecular weight (Li *et al.*).[40] There was an increase in lignin and silica percentage in both feedstocks treated with *Delftia* sp.

The percentage change in lignin observed in *Delftia* sp. supplemented paddy straw was 0.3%. The percentage change in silica was 5.5% in feedstock treated with *Delftia* sp. Lignin and silica are the non-degradable part of the matter. During anaerobic digestion, most of the cellulose and hemicelluloses are degraded by microbial attack, while lignin and silica are resistant to degradation and remain constant, but their percentage decreases as cellulose and hemicelluloses degrade.

CONCLUSION

The present study concluded that *Delftia* sp. are potent bacteria for lignocellulolytic enzyme production using biodigested slurry as a substrate for SmF. *Delftia* provides shorter incubation time, higher yield, less shear stress, homogeneity for its dispersal and increased yields of enzymes. Also, for most enzymes, the activity was maximum in 50% biodigested slurry, compared to paddy straw broth and nutrient broth, indicating that it is a suitable lignocellulosic substrate for enzyme production. Moreover, the biogas production increased in the *Delftia* enzyme enriched paddy straw plant, as compared to the control, with no enzyme enriched slurry, by 35%. This may be attributed to the decrease in the percentage of total solids, volatile solids, cellulose, hemicelluloses and lignin.

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