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Optimization of cultural conditions for submerged state fermentation of digested biogas slurry for production of lignocellulolytic enzymes using *Phanaerochaete chrysosporium* MTCC 787

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Abstract: Growing environmental concerns and increasing demands from end-use sectors have increased the global market for microbial products. Optimizations of production parameters hold great importance for the industry. The present study was aimed at optimization of submerged state fermentation conditions for production of lignocellulolytic enzymes from digested biogas slurry by *Phanaerochaete chrysosporium* MTCC 787. Enzyme activities for different enzymes i.e. endoglucanase, exoglucanase, β-glucosidase; xylanase and mannanase; laccase, lignin peroxidase and manganese peroxidise, using *P. chrysosporium* MTCC 787 were maximum at 50% concentration of digested slurry and showed maximum value of xylanase i.e. 187.41U/ml. Effect of temperature (25°C, 30°C and 35°C) on lignocellulosic bioconversion showed that at 30°C, maximum value of manganese peroxidise (167.5 U/ml) was obtained. Highest enzyme activites were obtained at selected inoculum size i.e. 10^7spores/ml, e.g. 85.29 U/ml xylanase was obtained. Incubation period of 8 days and pH of 7.0 came out to be best conditions for *P. chrysosporium* MTCC787 to produce maximum enzyme activity e.g. xylanase 95.47U/ml at pH 7.0 and xylanase 144.96U/ml at 8 day incubation.This work presents a novel concept in optimization of fermentation process to produce lignocellulolytic enzymes as this work is focussed on utilization of digested biogas slurry as a substrate for enzyme production and enhancement of the production with microbial source, which is environment friendly.

Keywords: Cultural conditions, Digested biogas slurry, Enzymes, Fungi, Lignocelluloses, Optimization

INTRODUCTION

In the modern era, the technologies related to microbial production of biomolecules like enzymes, antibiotics, metabolites and polymers etc. have matured to a greater extent. Microbes are being used for commercial production of a wide variety of products such as fertilizers in agrochemical sector, biopharmaceuticals and therapeutics in the healthcare sector, biopolymers and biofuels in the energy and environment sectors.

Fermentation is a very versatile process technology for producing such value added products and since fermentation parameters have a high impact upon the viability and economics of the bioprocess, their optimization holds great importance for process development. Especially, the microbial enzyme production is greatly influenced by fermentation conditions such as pH, temperature, substrate concentration and agitation as well as by the incubation period. Moreover, besides the fermentation conditions, the chemical structure, monomer composition, and physicochemical and rheological properties of the final product also change with the type of strain. This in turn allows the industrial production of enzymes with desired specifications via controlling the fermentation conditions, choosing feasible feedstocks, and using high-level producer strains (McWilliams, 2011).

Fermentation feedstock can represent almost 30% of the cost for a microbial fermentation. Thus, to maximize the cost effectiveness of the process, recent work has shifted to use cheaper alternatives such as olive mill wastewater (OMW), syrups, and molasses as the substrate (Robinson and Nigam, 2001). Lignocellulosic biomass is a cheap and abundant alternative for microbial biopolymer production (Ding and Himmel, 2006; Lee, 2005). Thus, the presented work is focussed on using digested biogas slurry, which is a cheaper and easily available feedstock for fermentation.

Fungi possess an efficient hydrolytic system capable to convert lignocellulosic material to essential metabolites for growth (Khalid *et al.,* 2006). Usually, these fungi secrete enzymes, including cellulases (cellobiohydrolases, endoglucanases), hemicellulases (xylanases) and β-glycosidases. Mutant strains of *Trichoderma reesei* have been selected that produce extracellular cellulases up to 35 g/l (Jorgensen *et al.,* 2003).

Cellulases hydrolyze cellulose to smaller sugar components like glucose and are used in the production of

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fermentable sugars, ethanol, organic acids, detergents, pulp and paper industry, textile industry and animal feed (Chellapandi and Himanshu, 2008; Karmakar and Ray, 2010; Omosajola and Jilani, 2008b). Cellulases can be divided into three major enzyme activity classes; endoglucanses or endo1,4β-glucanase (EC 3.2.1.4), cellobiohydrolase (EC 3.2.1.91) and β-glucosidase (D-glucoside glucohydrolase) (EC 3.2.1.21) (Han *et al*., 2009). The cellulase systems of the mesophilic fungi *Trichoderma reesei* and *Phanerochaete chrysosporium* are the most thoroughly studied (Kaur *et al.,* 2007).

Hemicellulose are heterogeneous polymers built up by pentoses (D-xylose, D-arabinose), hexoses (D-mannose, D-glucose, D-galactose) and sugar acids. There are various enzymes responsible for the degradation of hemicelluloses like endo-1,4-β-xylanase, β-xylosidase, β-glucuronidase and acetylxylan esterase, β -mannanase and β-mannosidase etc. Xylanases are being used as additives in feed for poultry and as additives to wheat flour (Rahman *et al.,* 2007).

Lignin degradation is known to be performed by most of the white-rot fungus like *Ceriporiopsis subvermispora*. Most of the research concerning biodegradation of lignin has been focused on *Phanerochaete chrysosporium*, *Streptomyces viridosporous, Pleurotous eryngii, Trametes trogii and Fusarium proliferatum* (Regalado *et al*., 1997)

Keeping in view, the present study was aimed to optimize cultural and biochemical parameters for lignocellulolytic enzyme production from *P.chrysosporium* MTCC 787.

MATERIALS AND METHODS

Procurement of digested biogas slurry, microbial cultures and chemicals: Digested biogas slurry was procured from a working biogas plant in biogas field laboratory of School of Energy Studies for Agriculture (Punjab Agricultural University), Ludhiana.Standard fungal culture*Phanerochaete chrysosporium* MTCC 787 was procured from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh. The culture was maintained on potato dextrose agar media slants at 30±2^{0}C and was stored in refrigerator after sub-culturing. Chemicals used for solutions preparation for enzymatic analysis were of analytical grade and were purchased from Hi-Media, SRL, Sigma and S.D fine chemicals Pvt. Ltd.

Enzymatic analysis of digested biogas slurry: Hundred milliliter of digested biogas slurry was taken in triplicate test tubes each. Hundred ml of distilled water was added in each test tube containing slurry and was properly mixed. The samples were centrifuged at 10,000 rpm for 15 minutes at 4°C to get clear supernatant. The supernatant was used as crude enzyme extract and was analyzed for activities of endoglucanase and exoglucanase by Mandels *et al* (1976) method. Dinitro-salicylic acid (DNS) method was used for the analysis. The β-glucosidase activity was measured by the methods of Toyama and Ogawa (1977) and protein content by Lowry *et al* (1951) method. Lignolytic enzyme laccase was determined by the method of Turner (1974) with some modifications described by Singh *et al* (1988). Lignin peroxidase activity was determined by the method given by Tien and Kirk (1988). Manganese peroxidase activity was determined by method of Paszczynski *et al*(1988). Xylanase activity was measured using the method of Singh *et al* (2000) and Mannanase activity was measured by Growindhagaer *et al* (1999). Enzyme activities (U/ml of sample) and protein (mg/ml of sample) was determined spectrophotometrically using UV-VIS spectrophotometer 2800 model.Reducing sugars produced by cultures were estimated by Miller's (1959) dinitro-salicylic acid (DNS) method.

The enzyme activity was expressed in terms of International units, which is defined as 1 micromole of reducing sugar released per mililitre of enzyme extract, measured as glucose or xylose or mannose. Appropriate dilution factors were used as and when followed during estimation of enzyme activity.

Optimization of process parameters for production of industrial enzymes: Various parameters viz. slurry concentration (25-75%), spore concentration (10^6-10^9 spores/ml), pH (4-9), incubation temperature (25-35° C) and varying incubation period (4-16 days) were studied for optimization of ligno-cellulolytic enzymes from *P.chrysosporium* MTCC 787 using digested biogas slurry as a substrate.For the optimization of slurry concentration, digested biogas slurry mixture (DBS and water) was taken in different concentrations i.e. 25%, 50% and 75% and were inoculated with 10^7spores/ml of *P.chrysosporium* MTCC 787, incubated at 30±2°C for 5 days. The effect of spore concentration was studied by inoculating the diluted digested biogas slurry (50%) with inoculum of different sizes of *P.chrysosporium* MTCC 787 i.e. 10^6,10^7 and 10^8 spores/ml spore suspension and incubated at 30±2°C for 8 days in a BOD incubator. For pH, the initial pH of the substrate (digested biogas slurry) was set to 4.0, 5.0 6.0, 7.0, 8.0 and 9.0 by the addition of buffer solutions of varying pH and inoculated with mycelia bits (5mm dia) of *P.chrysosporium* MTCC 787, incubated for 8 days. To study the effect of incubation period, diluted digested biogas slurry (50%) inoculated with the 7 day old culture (10^7 spores/ml) was incubated for different incubation period i.e. 4, 8,12 and 16 days at 30±2ºC in a BOD incubator. For, incubation temperature, the inoculated flasks of diluted digested biogas slurry of pH 7.0 were incubated at different temperature starting from 20 to 35°C with 5°C intervals i.e 20, 25, 30 and 35°C for 8 days in a BOD incubator.

For analysis of enzyme activities, the crude enzyme was

extracted by centrifugation and enzyme activities were assayed.

RESULTS AND DISCUSSION

Various cultural conditions like concentration of slurry, incubation temperature, inoculums size, incubation period and pH etc. affect the production of enzymes. The effect of these parameters on production of lignocellulolytic enzymes was studied using *Phanaerochaete chrysosporium MTCC 787*for optimization studies using one variable at a time approach. The results are discussed under following subheads:

Effect of slurry concentration: For optimizing the fermentation processes, the concentration of the substrate plays an important role. It was observed that in digested biogas slurry, enzyme activities for different enzymes i.e. endoglucanase, exoglucanase, β-glucosidase; xylanase and mannanase; laccase, lignin peroxidase and manganese peroxidase were maximum at 50% concentration (Table 1). *Phanaerochaete chrysosporium* MTCC 787 showed higher lignocellulolytic enzyme production i.e. endoglucanase (1.36U/ml), exoglucanase (0.894U/ml), β-glucosidase (4.377 U/ml); xylanase (187.41U/ml) and mannanase (85.0U/ml); laccase (12.0U/ml), lignin peroxidase (10.83U/ml) and manganese peroxidase(58.34 U/ml).

Fungi prefer a moist environment for their growth as it influence cell growth, the biosynthesis and secretion of enzymes. Lower moisture content causes the reduction in the solubility of the substrate nutrients, low degree of swelling and high water tension (Moo-young *et al.,* 1985; Lon sane *et al.,* 1985). Kundu *et al* (1983) observed that moisture level for solid state culture below the determined optimal value, leads to enzyme inhibition, whereas above the optimum level, greater enzyme diffusion away from the substrate take place. Laukevics *et al* (1984) also observed that very little moisture inhibits the growth and enzyme activity of the fungi and also the accessibility to nutrients, while very high moisture compact the substrate, prevent oxygen penetration and facilitates contamination by fast growing bacteria. Fungus growing at lower water ratio offers significant advantage in reducing the risk of contamination, since most bacterial species are unable to grow at reduces moisture level (Kheng and Omar, 2005). However, the optimum moisture level varies with the substrate used, as the various type of substrate have different water holding capacity.

Higher moisture content can cause a reduction in the enzyme yield due to its steric hinderance to cell growth of the enzyme producing strain which results from the reduction in the solid matrix porosity (interparticle spaces). Less porosity interferes with oxygen transfer and in turn influences the cell growth. Maurya *et al* (2012) reported that the maximum yield of enzyme (2.29 U/ml) was obtained at 70% moisture level. However, Mekala *et al* (2008) reported that at high moisture level (70%), the substrate prevents oxygen penetration and facilitates the contamination, whereas the low moisture level inhibits the growth, enzyme activity and accessibility to nutrients. An increase in the initial moisture content of substrate from 55-74% greatly enhanced the enzyme activity of the broth. However, a further increase to 80% had a negative effect on the production of the cellulolytic enzymes (Jecu, 2000).

Effect of incubation temperature: Temperature has a profound effect on lignocellulosic bioconversion. Results from Table 2 showed that *Phanaerochaete chrysosporium MTCC 787*showed maximum enzyme production at 30°C. The maximum activities of endoglucanase (0.997U/ml), exoglucanase (0.785U/ml), β-glucosidase (4.321U/ml); xylanase (128.12U/ml), mannanase (72.5U/ml), laccase (6.67U/ml), lignin peroxidase (32.5U/ml) and manganese peroxidase(167.5 U/ml) were recordedat 30°C.

Some of the thermophilic fungi, having maximum growth at or above 45–50 °C had produced lignocellulases with maximum activity at 50–78 °C (Wojtczak *et al.,* 1987). The temperature for assaying cellulase activities are generally within 50–65 °C for a variety of microbial strains e.g. *Thielavia terrestris*-255, *Mycelieopthora fergussi*-246C, *Aspergillus wentii, Penicillum rubrum*, *Aspergillus niger, Aspergillus ornatus* and *Neurospora crassa* (Menon *et al* 1994, Rajendran *et al* 1994, Steiner *et al* 1993), whereas growth temperature of these microbial strains was found to be 25–30 °C (Macris *et al.,* 1989).

Effect of spore concentration: For efficient production of lignocellulolytic enzymes, proper inoculum size is required. Results from Table 3 showed that for *Phanaerochaete chrysosporium MTCC* 787, maximum enzyme activities were found with 10□ spores/ml inoculums.

Results from Table 3 indicate highest endoglucanase activity by *Phanaerochaete chrysosporium MTCC* 787(0.787U/ml) at 10□ spores/ml spore concentration. Maximum xylanase activity (85.29 U/ml) was observed in *Phanaerochaete chrysosporium MTCC* 787 at 10□ spores/ml spore concentration.

Effect of pH: pH is an important factor affecting enzyme production (Pardo and Forchiassin, 1999). Results from Table 4 showed the effect of varying pH on the production of industrial enzymes from fungal culture. At pH 7.0 *Phanaerochaete chrysosporium MTCC* 787 showed maximum exoglucanase (0.658 U/ml), xylanase (95.47U/ml), mannanase (7.0 U/ml) and lignin peroxidase3 (7.83 U/ml).

The effect of pH on cellulase production was analysed using *Aspergillus niger* by Menon *et al* (1994) and it was reported that pH 5.5 was optimal for maximum cellulase production, while the pH range of 5.5–6.5 was optimal for β-glucosidase production from *Penicillium rubrum*. Eberhart *et al* (1977) had reported that

Table 1. Effect of slurry concentration on enzyme production from digested biogas slurry inoculated with mesophilic culture *Phanaerochaete chrysosporium* MTCC 787.

Slurry Concentration (%)	Enzyme activity (U/ml)							
	Endoglucanase	β-glucosidase	Exoglucanase	Xylanase	Mannanase	Laccase	Manganese Peroxidase	Lignin Peroxidase
25	1.354 ± 0.293	4.356 ± 0.292	0.788 ± 0.144	115.48 ± 0.599	62.0 ± 0.577	9.0 ± 0.881	48.35 ± 0.292	3.67 ± 0.619
50	1.367 ± 0.293	4.377 ± 0.290	0.894 ± 0.145	187.41 ± 0.593	85.0 ± 0.576	12.0 ± 0.881	58.34 ± 0.588	10.83 ± 0.640
75	1.094 ± 0.318	4.363 ± 0.289	0.819 ± 0.146	151.28 ± 0.584	10.0 ± 0.577	3.33 ± 0.485	31.7 ± 0.296	5.83 ± 0.640

#Cultural conditions: Incubation period: 5 days; Incubation temperature: 30±2°C; Spore concentration: 10^7 spores/ml of suspension; pH: 6;

Table 2. Effect of incubation temperature on enzyme production from digested biogas slurry inoculated with *Phanaerochaete chrysosporium* MTCC 787.

Spore concentration (spores/ml)	Enzyme activity (U/ml)							
	Endoglucanase	β-glucosidase	Exoglucanase	Xylanase	Mannanase	Laccase	Manganese Peroxidase	Lignin Peroxidase
10^6	0.660±0.061	4.373±0.291	0.630±0.058	79.33±0.587	66.5±0.600	7.67±0.619	6.67±0.619	7.83±0.115
10^7	0.787±0.064	4.496±0.290	0.756±0.060	85.29±0.585	74.5±0.600	15.64±0.615	8.34±0.588	9.34±0.588
10^8	0.664±0.061	4.413±0.029	0.667±0.061	74.06±0.577	65.5±0.600	14.67±0.619	5.0±0.577	6.67±0.619

#Cultural conditions: Incubation period: 5 days; Slurry concentration: 50%; Spore concentration: 10^7 spores/ml of suspension; pH: 6

Table 3. Effect of spore concentration on enzyme production from digested biogas slurry inoculated with *Phanaerochaete chrysosporium* MTCC 787.

Spore concentration (spores/ml)	Enzyme activity (U/ml)							
	Endoglucanase	β-glucosidase	Exoglucanase	Xylanase	Mannanase	Laccase	Manganese Peroxidase	Lignin Peroxidase
10^6	0.660±0.061	4.373±0.291	0.630±0.058	79.33±0.587	66.5±0.600	7.67±0.619	6.67±0.619	7.83±0.115
10^7	0.787±0.064	4.496±0.290	0.756±0.060	85.29±0.585	74.5±0.600	15.64±0.615	8.34±0.588	9.34±0.588
10^8	0.664±0.061	4.413±0.029	0.667±0.061	74.06±0.577	65.5±0.600	14.67±0.619	5.0±0.577	6.67±0.619

#Cultural conditions: Incubation period: 5 days; Slurry concentration: 50%; Incubation temperature: 30±2°C; pH: 6

Table 4. Effect of pH on enzyme production from digested biogas slurry inoculated with mesophilic culture *Phanaerochaete chrysosporium* MTCC 787.

pH	Enzyme activity (U/ml)							
	Endoglucanase	β-glucosidase	Exoglucanase	Xylanase	Mannanase	Laccase	Manganese Peroxidase	Lignin Peroxidase
5	0.605±0.115	4.376±0.590	0.306±0.057	29.48±0.599	1.5±0.288	4.5±0.600	7.06±0.577	2.5±0.600
6	0.640±0.116	4.312±0.586	0.596±0.066	38.61±0.612	2.0±0.577	2.34±0.059	8.34±0.588	3.34±0.588
7	1.371± 0.062	4.378±0.590	0.658±0.060	95.47±0.598	7.0±0.577	7.87±0.646	8.67±0.619	7.83±0.115
8	1.043±0.438	4.368±0.598	0.603±0.057	48.12±0.355	5.0±0.577	3.67±0.619	7.34±0.588	6.34±0.588

#Cultural conditions: Incubation period: 5 days; Slurry concentration: 50%; Incubation temperature: 30±2°C; Spore concentration: 10^7

production and release of cellulase depended on the pH of the medium. His observations indicated that extracellular release of cellulase from *Neurospora crass* occurred at pH 7, whereas the enzyme remained accumulated in the cell at pH 7.5. Similarly, pH 7 was suitable for extracellular production of cellulase from the *Humicola fuscoatra* (Rajendran *et al*., 1994). Further, the adsorption behaviour of cellulases was also affected by the pH of the medium (Kim *et al.,* 1988; Mukhopadhyey and Nandi, 1999).Lignolytic enzymes like

Table 5. Effect of incubation period on enzyme production from digested biogas slurry inoculated with mesophilic cultures.

Incubation period	Enzyme activity (U/ml)							
	Endoglucanase	β-glucosidase	Exoglucanase	Xylanase	Mannanase	Laccase	Manganese Peroxidase	Lignin Peroxidase
4	0.748± 0.059	5.054±0.577	0.772±0.062	110.21±0.581	55.5±0.600	2.67±0.399	7.97±0.661	1.78±0.303
8	0.784±0.073	5.290±0.580	0.785±0.060	144.96±0.869	58.5±0.600	7.43±0.594	12.65±0.616	4.89±0.316
12	0.779±0.066	5.225±0.582	0.635±0.029	136.89±0.649	46.5±0.600	7.41±0.593	11.56±0.606	4.87±0.646
16	0.766±0.058	5.144±0.599	0.483±0.060	133.73±0.649	48.0±0.600	6.67±0.594	11.08±0.625	3.68±0.649

#Cultural conditions: Slurry concentration: 50%; Incubation temperature: 30±2°C; pH: 8; Spore concentration: 10^7

laccase showed pH optimum at neutral pH, both unusual properties for most known fungal laccases and with guaiacol and syringaldazine the pH optima were rather broad: 5–7.5 and 6–7, respectively (Dhakar and Pandey 2013). The optimum pH for laccase production from *Trametes hirsuta* MTCC 11397 was between 5.5 and 7.5. (Baldrian 2006; Jordaan and Leukes 2003).

Effect of incubation period: Incubation period plays an important role in substrate utilization and enzyme production. Maximum activity of enzymes was obtained at 8 day of incubation in most of the cultures (Table 5).Out of all the enzymes analyzed, xylanase (144.96U/ml) showed highest activity in *Phanaerochaete chrysosporium* MTCC 787 after 8 days of incubation.

Conclusion

Submerged state fermentation of digested biogas slurry is a novel idea for production of lignocellulolytic enzymes, which provides a clean and green energy technology. This production is enhanced with the use of fungus *Phanaerochaete chrysosporium* MTCC 787 at 50% concentration of slurry, 30 °C incubation temperature, 10□ spores/ml of inoculum size, 8 day of incubation period and 7 pH etc. This technology if used commercially can be an energy efficient way of utilizing the digested biogas slurry and will add to its value.

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