

Production of Xylanase and CMCase by *Pleurotus ostreatus* in Polyurethane Foam based Solid State Fermentation

Kausik Majumder*

Department of Botany, Bidhannagar College, Government of West Bengal,
EB-2, Sector-1, Salt Lake City, Kolkata- 700 064, West Bengal, India,

Abstract

Tamarind kernel powder (TKP), an agro-residue was employed to evaluate the production of both xylanase and cellulase enzymes in polyurethane foam (PUF) based solid state fermentation by *Pleurotus ostreatus*, an edible mushroom. Soluble substrate TKP containing xyloglucan as major polysaccharide induced both xylanase and cellulase enzymes and enzyme production increased upto 3 % (w/w) TKP with culture filtrate consisting of xylanase and CMCase at a ratio of 2:1 app. Presence of sucrose (5 g/l) in TKP medium increased xylanase activity (118.3 U/ml) although CMCase activity was more or less unaffected. CMCase activity was significantly increased when the culture media containing mixed substrate i.e., both TKP and cellulose. The present study reports the successful utilization of tamarind kernel powder, an abundantly available soluble agro-residue for the enzyme production. It may also be concluded that solid state fermentation with polyurethane foam as an inert support is a promising approach for the production of enzymes for the future up-scaling process.

Keywords: Tamarind kernel powder, *Pleurotus ostreatus*, CMCase

1. Introduction

Advances in industrial biotechnology offer potential opportunities for economic utilization of agro-industrial residues and in recent years there has been an increasing trend towards more efficient utilization of agro-industrial residues. The enzymatic hydrolysis of xylans and cellulose has been extensively reviewed and out of the all xylanolytic and cellulolytic enzymes, the *endo* 1,4 β -D xylanase (EC 3.2.1.3) and *endo* 1,4 β -D glucanase (EC 3.2.1.4) are the best characterized enzymes. There are many reports available on the production of these enzymes by submerged fermentation or solid state fermentation using a wide variety of carbon substrates (Botella et al., 2007) although edible

mushroom is not effectively exploited for the enzyme production process. Moreover, use of soluble carbon substrate is a prerequisite for development of an effective enzyme production system.

Tamarind kernel powder (TKP), obtained from tamarind (*Tamarindus indica*) seed, an agro-residue accumulated in huge amounts and the major polysaccharide present in it is xyloglucan having β (1,4) linked D-glucose backbone with D-xylose, D-galactose and L-arabinose present in the side chain (Gidley et al., 1993). In our previous study, we have shown that TKP induced all cellulolytic and xylanolytic enzymes in either submerged fermentation (Chatterjee et al., 2010) or in solid state fermentation (Majumder et al., 2015) by *Termitomyces clypeatus*, an edible mushroom. However very few reports are on the record regarding the co-production of xylanase and cellulase by *Pleurotus ostreatus* in solid state fermentation (Quinnghie et al., 2004; Khalil et al., 2011, Karthikeyan et al. 2015). The present set of researches has focused on the optimized production in polyurethane foam, an inert support based solid state fermentation utilizing utilizing the soluble property of TKP xyloglucan, which is a key parameter in SSF by *Pleurotus ostreatus*, for the future up-scaling process.

2. Material and Methods

2.1 Microorganism and fermentation system

Mycelial culture of *Pleurotus ostreatus* was grown at $30 \pm 1^{\circ}\text{C}$ for 5 days in medium containing 1 % glucose, 1 % malt extract, 10 % potato extract and 0.15 % KH_2PO_4 at pH 5.0. The enzyme production medium for solid state fermentation contained (g/l): $\text{NH}_4\text{H}_2\text{PO}_4$ - 24; MgSO_4 , 7 H_2O - 0.5; CaCl_2 , 2 H_2O - 0.37; H_3PO_4 - 0.57; FeSO_4 , 7 H_2O - 0.25; MnCl_2 - 0.032, NaMoO_4 - 0.032; yeast extract- 5 in combination with a carbon sources at different

concentration and the pH of the medium was adjusted to 5.0. Solid state fermentation was carried out according to the procedure of Majumder et al., (2015) using cubes of solid polyurethane foam (PUF) at $30 \pm 1^\circ\text{C}$ for 7 days.

2.2 Carbon substrates

Cellulose (Sigmacell, Type 50) was obtained from sigma Chemicals Company, USA. Tamarind kernel powder (TKP) was obtained from a local market. The powder was initially screened through 200 meshes to remove seed skin and the sieved mass was dried overnight at 60°C .

2.3 Enzyme activity assays

CMCase (carboxy-methyl cellulase,) and xylanase activities of the culture filtrate were measured in terms of *endo* 1,4 β -D glucanase (EC 3.2.1.4) and *endo* 1,4 β -D xylanase (EC 3.2.1.3) activities using carboxymethyl cellulose and oat spelt xylan as substrates respectively (Sengupta et al., 2000). Units of CMCase and xylanase activities were represented as μmoles of glucose or xylose equivalent liberated per minute under the assay conditions. Data presented for enzyme activities were mean values of data obtained from 5 sets of experiments under identical conditions.

3. Results and Discussion

3.1 Co-production of xylanase and CMCase in PUF supported culture

The extracellular enzyme production in solid state fermentation of *Pleurotus ostreatus* is represented in Figure 1 and it was observed that with increasing concentration of TKP, the extracellular enzyme i.e., both xylanase and CMCase activity in the culture filtrate increased with increasing input of carbon substrate concentration. The xylanase activity was found at 0.5 % conc. of TKP as 42.5 U/ml, while in the conc. of 4.0 % of TKP, xylanase activity was 85.3 U/ml. On the other hand, CMCase activity was much lower than xylanase production although, the concentration of TKP significantly induces the higher rate of enzyme synthesis. The results also evinced with increase of TKP concentration in culture media, the biomass level of the fermentation system also increased, although higher concentration of TKP i.e., more than 3.0 % did not significantly increase the biomass level of the fermentation system. The results also stated higher concentration of TKP could not be able to induce higher rate of protein synthesis as well as higher rate of enzymes secretion. The biomass growth and consumption of carbohydrate source is presented in Figure 2 and from the available findings, it could be noted at the end of the fermentation system i.e., after 7th day of fermentation, the residual carbohydrate was present

at a substantial level. The results pointed out that fungus absorbed or utilized the optimum carbon source and after that concentration, fungal biomass neither increased nor utilize the remaining carbon source. The xylanase and CMCase productivity of the biomass (Table 1) points out that 0.5 % TKP containing medium gave the yield coefficient as 26.56×10^3 , while 2 % TKP containing medium showed a value as 21.91×10^3 for xylanase enzyme. On the other hand, yield coefficient of CMCase was 11.43×10^3 and 10.88×10^3 in 0.5 % and 2 % TKP medium respectively.

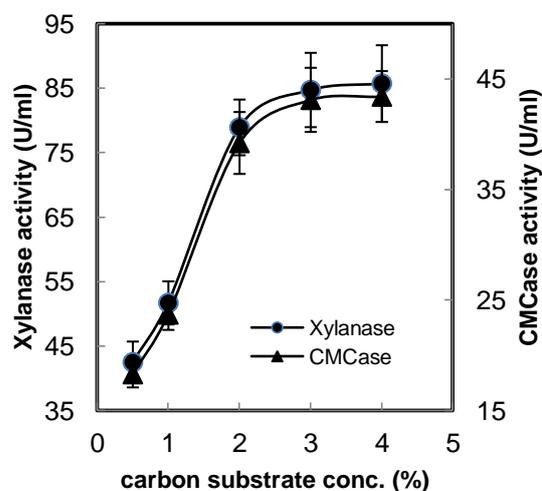


Figure 1: Production trend of enzyme titers (U/ml) as xylanase (*endo*-1,4 β -D xylanase) and CMCase (*endo*-1,4 β -D glucanase) influenced by the carbon substrate conc. as TKP by *Pleurotus ostreatus* in solid state fermentation. Data are presented here the mean value of five identical set of experiments with standard error bar.

Enzyme production is a growing field of biotechnology and most enzyme manufacturer produce enzymes using submerged fermentation technique (Atlaf et al., 2010). Agro-industrial residues are generally considered the best substrate for SSF bioprocess, especially for enzyme production (Getachew et al. 2016). However, SSF of fungi on insoluble substrate did not find success industrially because of complexity in downstream processing. Solid state fermentation cultured on an inert support like PUF is a more advanced technique over SSF on natural substrate since it improves control of heat and mass transfer during fermentation with a less complicated product recovery and enzyme processing (Ooiyass et al., 2000). This process has however been less attempted in enzyme production, possibly because of the unavailability of suitable soluble substrate and thus searching for a novel soluble carbon source is a prerequisite for the enzyme production system.

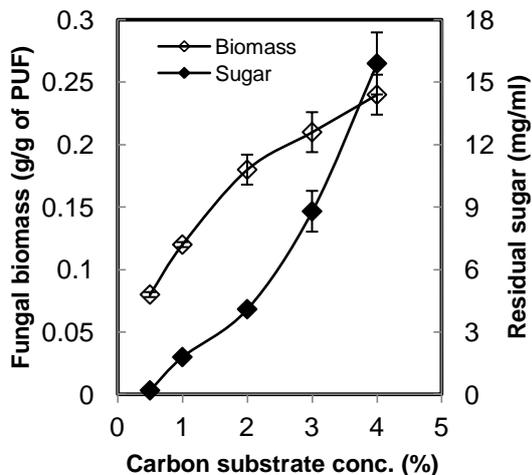


Figure 2: Biomass production and substrate consumption as residual sugar present in the culture filtrate of *Pleurotus ostreatus*, grown on PUF in solid state fermentation with varying degree of initial carbon substrate concentration. Data are presented here the mean value of five identical set of experiments with standard error bar.

Table 1: Yield coefficient ($Y_{E/x}$) enzyme yield/ g of biomass as influenced by the carbon substrate conc. as TKP by *Pleurotus ostreatus* in PUF based solid state fermentation.

Yield coefficient ($Y_{E/x}$) x 10^3 enzyme yield/ mg of PUF		
Carbon substrate conc. (%)	Xylanase	CMCase
0.5	26.56	11.43
1.0	21.54	9.87
2.0	21.91	10.88
3.0	20.16	10.26
4.0	17.17	9.1

In the SSF system, higher concentration of substrate reduced the fungal growth, related with high amount of residual carbohydrate was present at the end of fermentation. The correlation analysis provided results that substrate consumption and fungal biomass growth in solid state fermentation is highly related with each other at a linear function as $y = 0.15x + 0.996$ with a regression coefficient $r^2 = 0.985$ (Figure 3). A critical analysis of data revealed that xylanase production was significantly increased as 21.64 % and 85.6 % in the medium containing 1 % and 2 % TKP over 0.5 % TKP containing medium. Similar finding was observed in xylanase and cellulase production by *Termitomyces clypeatus* in submerged fermentation in which, culture filtrate for 3 % TKP consisting of xylanase and CMCase at a ratio of 4:1 approx. (Chatterjee et al., 2010). Elisashvili et al., (2006) reported that *Pleurotus dryinus* is able to produce cellulase and xylanase in submerged fermentation of mandarin peels, while *Pleurotus ostreatus* was found to be more efficient in

the cellulase production than *Pleurotus sajor-caju* in SSF using saw dust, sugarcane bagasse and paddy straw as substrate (Khalil et al., 2011). Although cellulase and xylanase were found to be coproduced by fungi, but relative production of two sets of enzymes were significantly influenced by the carbon substrate present in medium and also differed among the fungal strains used.

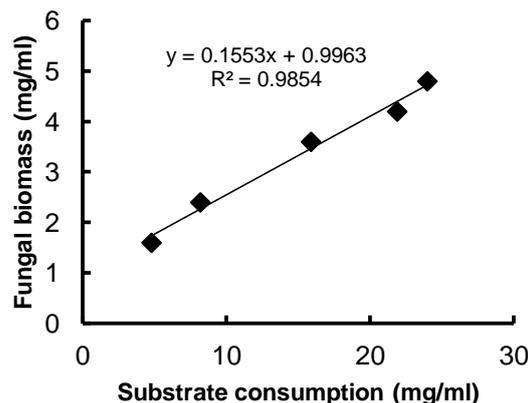


Figure 3: Correlation between biomass production and substrate consumption in PUF based solid state fermentation. Line correspond to a linear function as $y = 0.15x + 0.996$ with $r^2 = 0.985$

Thus the experiments clearly point out that TKP is a good option for enzyme production in solid state fermentation. Elisashvili et al., (2008) reported that *Pleurotus* sp. produced a variety of lingo-cellulosic enzymes in solid state fermentation, although the yield of xylanase (84.1 Uml^{-1}) and CMCase (62.3 Uml^{-1}) were much lower as compared to laccase production (4103 Uml^{-1}). Xyloglucan which contain xylose substituted β -1,4 glycosidic backbone could possibly released inducer structurally similar to those obtained during the simultaneous hydrolysis of cellulosic and hemi-cellulosic fraction of agro-residues. It may be opined that liberation of soluble inducer from cellulosic particles required synergistic participation of all cellulolytic enzymes assembled on the same particle. In presence of low basal level synthesis of enzymes, possibly for the rational adsorption of all enzymes uniformly on the same particle and consequent release of soluble inducer decreased at higher concentration of insoluble substrates. (Chatterjee et al., 2010). Menezes et al. (2010) also reported that *Pleurotus tailandia* produces a xylo-oligosaccharides and a number of simple sugars like xylose, arabinose, cellobiose, mannose and maltose after submerged fermentation for 40 days using oat spelt xylan as carbon substrate.

3.2 Effect of sucrose on the extra-cellular production of enzymes

Sucrose, a constitutive enzyme improves the stability of extra-cellular enzymes by formation of enzyme aggregates in culture filtrate. It would thus be necessary to determine whether and how far the degree of enzyme production as well as stabilization is influenced by sucrose present in the medium. It was observed that from Figure 4, that sucrose at 0.1 % conc. sucrose produced 76.6 U/ml of xylanase, while 0.5 % of sucrose produced 118.3 U/ml of xylanase activity as compared to 75.4 U/ml of xylanase, production had been found at the medium containing only 2 % TKP. On the other hand, 35.4 U/ml of CMCase has been noticed as compared to 36.3 U/ml and 48.7 U/ml of CMCase at the medium containing 0.1 % and 0.5 % sucrose respectively with the TKP as carbon source. The study also pointed out that sucrose at increasing concentration improved xylanase and CMCase production, although xylanase production of fungus was significantly increased at a ratio of 2.4:1.0 as compared to CMCase production. Similar findings were also observed in solid state fermentation of xylanase production by *Termitomyces clypeatus* (Majumder et al. 2015). The available findings therefore, are of indicative of the fact that sucrose had played a major role on improvement of enzyme production in solid state fermentation. In the present study, however, CMCase activity was not significantly improved by addition of sucrose in medium and it may be due to catabolic repression, generated by easily hydrolysis of the disaccharides or related with pH fall of the medium. Thus it may be concluded that xylanase and CMCase production in solid state fermentation is regulated by a level of reducing sugars but a differential carbon catabolic product repression is involved.

3.3 Enzyme production in the presence of mixed substrates

Although addition of sucrose in media containing TKP improved xylanase production by the fungus as observed in previous experiment (Figure 4), but CMCase production more or less unchanged. In the context of producing both xylanase and CMCase in a single set of fermentation, use of mixed substrates appeared to be beneficial for production of both enzymes in good yield in a single fermentation. With various combinations of the carbon sources used, it appeared that (Figure 5) cellulose or sucrose in TKP medium specifically improved production of either cellulase or xylanase, although increase in enzyme activities was observed in different extent. The findings clearly shown that both xylanase and CMCase production were improved when sucrose (0.5 %) and cellulose (0.5 %) were added in a TKP (2.0 %) media. Using cellulose and TKP as carbon

source in single set of fermentation by *Termitomyces clypeatus* was reported to improve both of xylanase and CMCase activities in culture filtrate in either submerged (Chatterjee et al., 2010) or solid state fermentation (Majumder et al., 2015).

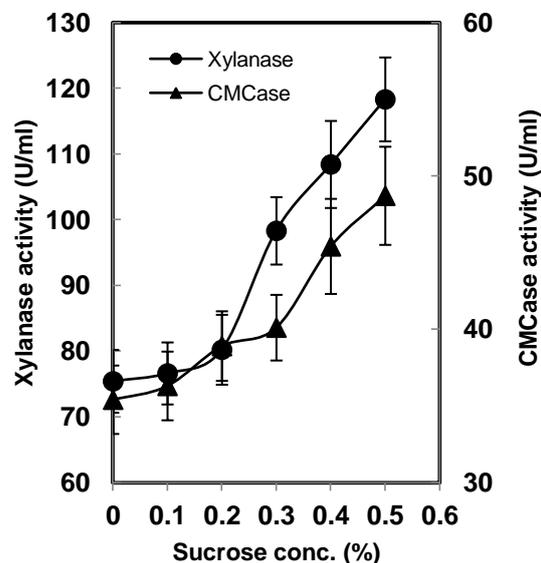


Figure 4: Production trend of enzyme titers (U/ml) as xylanase (*endo*-1,4 β -D xylanase) and CMCase (*endo*-1,4 β -D glucanase) influenced by varying level of sucrose with initial TKP conc. (2 %) by *Pleurotus ostreatus* in solid state fermentation. Data are presented here the mean value of five identical set of experiments with standard error bar.

However, Premkumar et al., (2018) reported that *Pleurotus florida* and *Pleurotus djamor* has obstructed on cellulase and xylanase production on mixed substrate (sugarcane bagasse mixed with wheat bran). From the experimental findings, it may be supposed that the inducer molecules liberated by TKP or cellulose were not identical. Both of the substrates induced CMCase and xylanase production independently. Thus the study showed that the production of xylanase and cellulase in solid state fermentation was not simultaneously regulated with fungal growth rather influenced by the carbon source in culture medium. Solid state fermentation on inert support with TKP media therefore facilitates a promising approach for industrial production of enzymes and future studies should be focused on critical physiological and kinetic analysis of process development, control strategies as well as downstream processing of inert support based solid state fermentation.

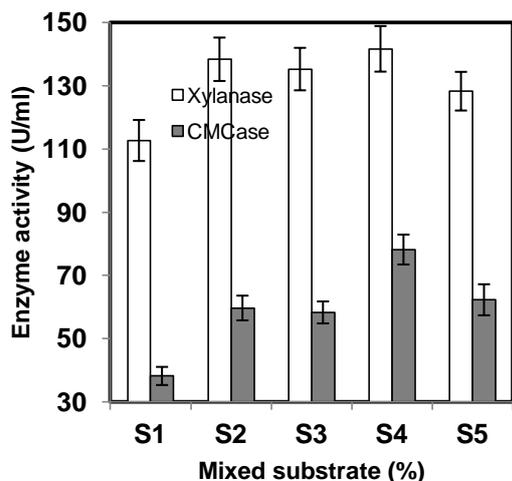


Figure 5: Production trend of enzyme titers (U/ml) as xylanase (*endo*-1,4 β -D xylanase) and CMCCase (*endo*-1,4 β -D glucanase) as influenced by varying levels of mixed carbon substrates in PUF based solid state fermentation by *Pleurotus ostreatus*. S1 = TKP (2%) + sucrose (0.5%), S2 = TKP (2%) + cellulose (0.5%), S3 = TKP (2%) + cellulose (1%), S4 = TKP (2%) + cellulose (0.5%) + sucrose (0.5%) and S5 = TKP (2%) + cellulose (1%) + sucrose (0.5%). Data are presented here the mean value of five identical set of experiments with standard error bar.

Conclusion

The choice of an appropriate substrate is of great importance for successful production of CMCCase and xylanase, and the cost of the substrate plays a crucial role in the economics of enzyme production. This work represents the information on CMCCase and xylanase production on an inert support based solid state fermentation by *Pleurotus ostreatus* using a soluble carbon substrate. However additional research would be necessary to characterize the enzymes and unfolding the regulation loops of enzyme production.

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