Hyperproduction of Manganese Peroxidase through Chemical Mutagenesis of *Trametes versicolor* IBL-04 and Optimization of Process Parameters

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This is the first report on chemical mutagenesis of *Trametes versicolor* IBL-04 to develop a hyper-producing mutant for overproduction of manganese peroxidase (MnP) using sugarcane bagasse as a substrate. A freshly prepared inoculum of indigenously isolated *T. versicolor* IBL-04 was treated with 100 µg mL^{-1} (*v/v*) ethyl methane sulfonate (EMS) and ethidium bromide (EB) separately for different time periods. The selected mutants and parent strain were cultured in solid-state fermentation (SSF) conditions to select the hyper-producing mutants. After selection of hyper-producing EMS- and EB-treated mutants, the fermentation parameters, including substrate type, incubation time, initial pH of the medium, temperature, moisture level, and carbon-to-nitrogen ratio (C:N), were optimized by adopting the Classical Optimization Strategy. *T. versicolor* IBL-04 treated for 90 min with EMS (EMS-90 mutant) gave maximum MnP production (935 U mL^{-1}) after 8 days of fermentation. Supplementation with carbon and nitrogen sources significantly enhanced mutant growth, and under optimum conditions, the maximum MnP production by the mutant strain increased to 3045 U mL^{-1}. The results indicated that the random chemical mutagenesis significantly enhanced the MnP production. The increased production of MnP by the EMS-90 mutant strain suggest its potential for commercial-scale enzyme production and biotechnological applications.

Keywords: Chemical mutagenesis; T. versicolor IBL-04; MnP; Agro-industrial residues; SSF

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INTRODUCTION

Enzyme production has been an area of research interest for the last few decades, but in recent years, enzyme biotechnologists have made tremendous progress in the area of industrial enzymology. Fungi are the most fascinating group of organisms, exhibiting great diversity, and the success of fungi for industrial enzyme production is largely due to their metabolic versatility. Fungi are well known producers of a wide variety of enzymes, organic acids, mycotoxins, and antibiotics. The importance of fungi is not limited to their native products; they are also useful in the development and commercialization of new products through the modern techniques of mutagenesis and molecular biology (Woolhouse and Gaunt 2007).

Lignin mineralizing enzymes, also known as wood-degrading enzymes, are extracellular and nonspecific enzymes that mainly include lignin peroxidase (LiP, EC 1.11.1.14), manganese peroxidase (MnP, EC 1.11.1.13), and laccase (EC 1.10.3.2), along with some accessory enzymes produced by white rot fungi (WRF) as secondary metabolites (Asgher *et al*. 2012b). MnPs are heme peroxidases that require H_2O_2 for their

activity (Cheng *et al*. 2007; Asgher and Iqbal 2013) and are attractive tools in biotechnological fields like bioremediation, biofuels, paper & pulp, and textile industries (Levin *et al*. 2008; Stoilova *et al*. 2010; Asgher *et al*. 2011a, 2012a). Various species of WRF, such as *T. versicolor*, *T. hirsuta*, and *T. ochracea*, have been reported to produce high titers of MnP along with LiP and laccase (Tomsovsky and Homolka 2003; Asgher and Iqbal 2011).

The demand for ligninolytic enzymes has increased in recent years due to their potential applications in diverse biotechnological processes (Crecchio *et al*. 1995; Wesenberg *et al*. 2003; Asgher *et al.* 2013; Asgher and Iqbal 2013; Iqbal and Asgher 2013). The ligninolytic enzyme titer of fungi is much higher than that from its bacterial counterparts, which could be the main reason for industrial biocatalysts' dependence on fungi. However, the yields, activities, and stabilities of these enzymes from indigenous WRF strains are not sufficient to meet industrial demands. Industrial strain improvement plays a central role in the development of commercial microbial fermentation processes. Mutant strains with enhanced enzyme production can be developed and screened on the basis of the overexpression of genes involved in enzyme production due to mutant regulatory genes (Fiedurek and Gromada 1997; Haq *et al*. 2004).

Mutagenic procedures can be optimized in terms of type of mutagen, dose level, and time of exposure to obtain industrially competent fungal strains (Rowlands 1984). However, in recent years, there have been no significant attempts at the overproduction of ligninolytic enzymes by inducing chemical mutagenesis. Strain improvement using mutagenesis and the selection of mutants is a time-consuming exercise, but it gives very encouraging results in most cases. In this study, the development of hyperproducing mutants of locally isolated *T. versicolor* IBL-04 by chemical mutagenesis was attempted for the overproduction of MnP.

EXPERIMENTAL

The mutagenesis and optimization studies were carried out in the Industrial Biotechnology Laboratory (IBL), Department of Chemistry and Biochemistry, University of Agriculture Faisalabad (UAF), Pakistan. All chemicals used were of analytical grade and were purchased mainly from Sigma-Fluka-Aldrich (USA).

Fungal Culture and Inoculum Preparation

Pure culture of the indigenously isolated strain *T. versicolor* IBL-04 was available at the IBL, UAF. Inoculum was prepared in 500-mL cotton-plugged Erlenmeyer flasks by growing the fungus in Kirk's basal medium (Tien and Kirk 1988). The medium was adjusted to pH 4.5 and sterilized (121 °C) for 15 min, and after cooling to room temperature, it was inoculated with *T. versicolor* IBL-04 slant culture. The inoculum flasks were incubated on an orbital shaker operating at 125 rpm (30±1°C) for 5 days to enable homogeneous spore suspension and its concentration was estimated using a haemocytometer (Kolmer *et al.* 1959) to get a homogenous inoculum containing $1x10^7$ spores mL^{-1} ((Kay-Shoemake and Watwood 1996).

Mutagenesis by EMS Treatment

A stock solution of 500 µg mL^{-1} (*v/v*) was used to prepare different EMS concentrations to treat the fungal cells. One mL of this solution was added separately to 9

mL of Vogel's medium containing fresh fungal spore inoculum (1×10^7 spores mL^{-1}). After a specific incubation time (30 to 120 min), the treated spores were harvested by washing with sterile biological saline solution (0.89% NaCl and 0.1% yeast extract) and centrifuging at 12,000 rpm for 10 min; this step was repeated three times to completely remove traces of EMS. The washed cells were spread on potato agar plates (PDA) and incubated at 30 °C for 4 to 6 days for colony formation.

Mutagenesis by EB Treatment

To prepare EB stock solution, 1.0 mg ethidium bromide was added per mL in ddH_2O, and from this stock solution, four further dilutions, of 25 µg mL^{-1}, 50 µg mL^{-1}, 75 µg mL^{-1}, and 100 µg mL^{-1}, were prepared and used in mutational work. One mL of EB added separately to 9 mL of Vogel's medium containing fresh fungal spore inoculum (1×10^7 spores mL^{-1}). After specified time internals (30, 60, 90, and 120 min), the treated spores were harvested by washing with sterile saline solution and were spread on agar plates for colony formation.

Colony Growth Restriction

Triton X-100 was used as a colony restrictor in PDA media (1% to 2%, *w/v*) to restrict the growth of fungal colonies (Khattab and Bazaraa 2005). A cell survival curve was constructed using a 3-log kill curve; doses producing 87% and 84% kills were considered to be the best using EMS and EB, respectively. Colony forming units (CFU) were calculated using the following formulation:

$$\text{CFU mL}^{-1} = \frac{\text{No of colonies appeared on plate}}{\text{Amount plated (0.1 mL)}} \times \frac{1}{\text{Dilution factor}}$$

Selection and Evaluation of Mutants

Mutation is a random event at the single cell level. 2-deoxy-D-glucose (2dDG) acts as a catabolic repressor and was used in this study for the selection of mutants as described by Gromada and Fiedurek (1997). It suppresses/inhibits the growth of parent spores, but mutant spores appear on PDA. The selected colonies were isolated and checked for MnP production.

Optimization of Culture Conditions

After selection of mutants, medium optimization was performed using the one-factor-at-a-time classical approach; evaluating the effect of one variable while others were maintained at a certain level. The following process parameters were optimized; incubation time (2, 4, 6, 8, 10, and 12 days) temperature (25 °C, 30 °C, 35 °C, 40 °C, and 45 °C), initial pH (3.0, 3.5, 4.0, 4.5, 5.0, 5.5, and 6.0), initial moisture content (40%, 45%, 50%, 55%, 60%, 65%, and 70%), glucose and ammonium nitrate as the C:N ratio (5:1, 10:1, 15:1, 20:1, and 25:1), and inoculum size (1 mL, 2 mL, 3 mL, 4 mL, 5 mL, 6 mL, and 7 mL). The effect of mediators (veratryl alcohol, $MnSO_4$, ABTS, oxalate, and H_2O_2) on MnP production by the selected mutants was also investigated under optimum conditions.

MnP Activity Assay

Enzyme activity was determined at the end of each optimization step. The culture supernatants were obtained after filtration and centrifugation (3,000 g). MnP was assayed

by the method of Wariishi *et al*. (1992). $MnSO_4$ was added to a sodium malonate buffer in the presence of H_2O_2, and absorbance was read at 270 nm (ε 270= 11590 M^{-1} cm^{-1}). MnP activities were expressed as U mL^{-1}.

Statistical Analysis

All experiments and enzyme assays were conducted in triplicate, and results are reported as means ±SE. The data were subjected to Analysis of Variance (ANOVA) under a Completely Randomized Design (CRD) (Steel *et al*. 1997).

RESULTS AND DISCUSSION

Mutagenesis by EMS Treatment

A dose rate of 100 µg mL^{-1} (*v/v*) of EMS mutagen was determined to be optimum for generating the 3-log kill curve (Fig. 1). It was noted that an exposure time of 90 min (EMS-90) gave the best results; subsequently, colonies appearing (Fig. 2) on petri plates containing 2-dDG were used for further work on optimization and enzyme production. The frequency of positive mutations and number of viable colonies decreases with an increase in mutagen dose rate (Petruccioli *et al*. 1995). Similarly, in the present study, as the time of exposure increased, the survival of the fungus decreased, with a decrease in viable colonies. The selected mutant was assigned the code EMS-90 and used for further studies.

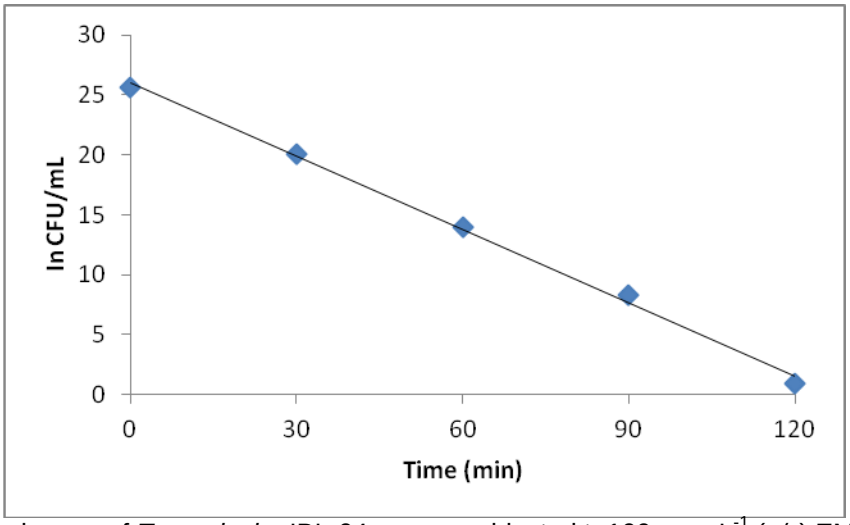

Fig. 1. Survival curve of *T. versicolor* IBL-04 spores subjected to100 µg mL^{-1} (*v/v*) EMS for varying time periods

Mutagenesis using EB Treatments

For the EB mutagen treatment, a 50-µg mL^{-1} (*w/v*) dose was determined to be optimum and gave the best results. A kill curve (Fig. 3) for EB-treated spores similar to that of EMS mutagenesis was prepared, and the time of exposure giving a 3-log kill was selected. The exposure time of 60 min was considered to be optimum, and colonies appearing on Petri plates (Fig. 4) were used for MnP production and process optimization.

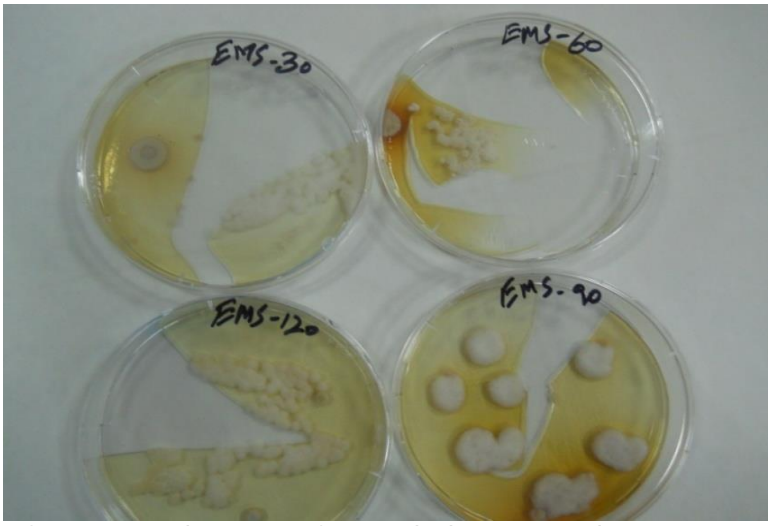

Fig. 2. Selection of EMS-treated mutants of *T. versicolor* IBL-04 using 2-DG as a selective marker

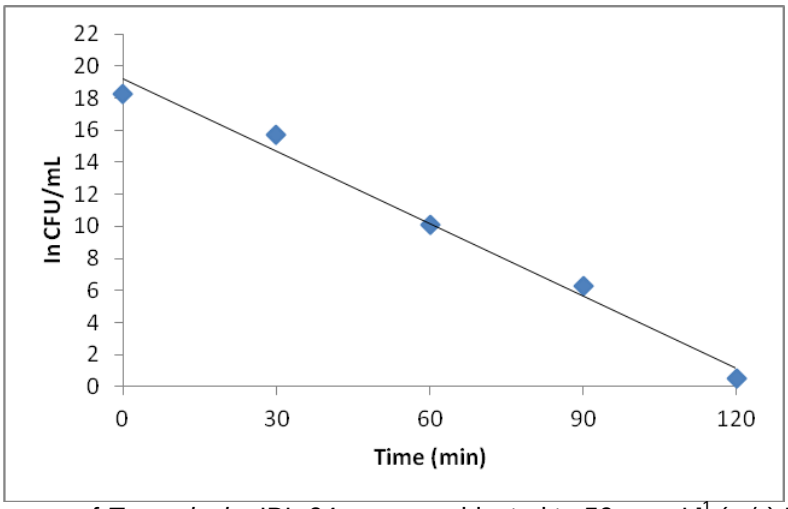

Fig. 3. Survival curve of *T. versicolor* IBL-04 spores subjected to 50 µg mL^{-1} (*w/v*) EB mutagen for varying time periods

Fig. 4. Selection of EB-treated mutants of *T. versicolor* IBL-04 on 2-DG as a selective marker

Production of MnP by Different *T. versicolor* IBL-04 Mutants

The results (Table 1) showed that the *T. versicolor* EMS-90 mutant was the best producer of MnP, followed by the EB-60 mutant, when grown on sugarcane bagasse in solid-substrate fermentation. Parent and selected mutant stains showed maximum enzyme activities on the eighth day of incubation. Among the various substrates investigated, sugarcane bagasse showed the best result, followed by rice straw and wheat straw in order. The nature and composition of the substrates used may be responsible for the increased growth of fungi on different substrates (Patel *et al*. 2009).The time taken by fungi for lignin-degrading enzyme synthesis is primarily dependent on the metabolism of a particular substrate and varies with the chemical composition of different lignocellulosic residues (Pandey *et al*. 2000).

Table 1. Production of MnP by *T. versicolor* IBL-04 Mutants using Different Ligninolytic Substrates

Mutagen Treatment	Mutants	Manganese Peroxidase activity (Mean ± S.E. U mL^{-1})			
		Sugarcane	Wheat straw	Rice straw	Corn cobs
NIL	Parent strain	580 ± 2.1	435 ± 5.6	513 ± 3.1	211 ± 3.5
Ethidium bromide (EB)	EB-30	109 ± 1.9	130 ± 3.1	219 ± 3.0	178 ± 2.9
	EB-60	624 ± 1.3	456 ± 3.4	543 ± 2.5	342 ± 1.6
	EM-90	451 ± 2.3	311 ± 4.8	479 ± 2.4	278 ± 4.0
	EM-120	309 ± 2.8	390 ± 2.5	265 ± 3.3	301 ± 1.1
Ethyl methyl sulphonate (EMS)	EMS-30	219 ± 2.2	231 ± 2.1	161 ± 2.1	201 ± 1.4
	EMS-60	389 ± 6.0	333 ± 4.7	300 ± 2.5	236 ± 2.4
	EMS-90	659 ± 3.1	289 ± 5.4	456 ± 2.1	342 ± 2.6
	EMS-120	356 ± 4.1	301 ± 7.9	209 ± 3.1	286 ± 1.2

Optimization of Culture Conditions

After the selection of EMS-90 and EB-60 as the best MnP-producing mutants, the enzyme production by the mutants was optimized. Different fermentation parameters were optimized using Classical Strategy: varying one parameter at a time and keeping the previously optimized ones at their optimum level.

Effect of Time Period

Culture was harvested for enzyme activity every two days. Maximum MnP was synthesized by the EMS-90 mutant (1017 U mL^{-1}), followed by the EB-60 mutant (733 U mL^{-1}) and the native strain (675 U mL^{-1}), after 8 days of incubation (Fig. 5). An increase in incubation time increased the enzyme activities during the first 8 days, and a further increase in the time period caused a decrease in enzyme activity. Zahmatkesh *et al*. (2010) recorded maximum MnP activity (471.7 U g^{-1}) on the 7^{th} day of *P. chrysosporium* cultivation, and Vaithanomsat *et al*. (2010) obtained optimum MnP activity after 8 days. The optimum incubation time-period for ligninolytic enzyme production by most WRF is 4-12 days (Bose *et al*. 2007; Asgher *et al*. 2008; Iqbal *et al*. 2011).

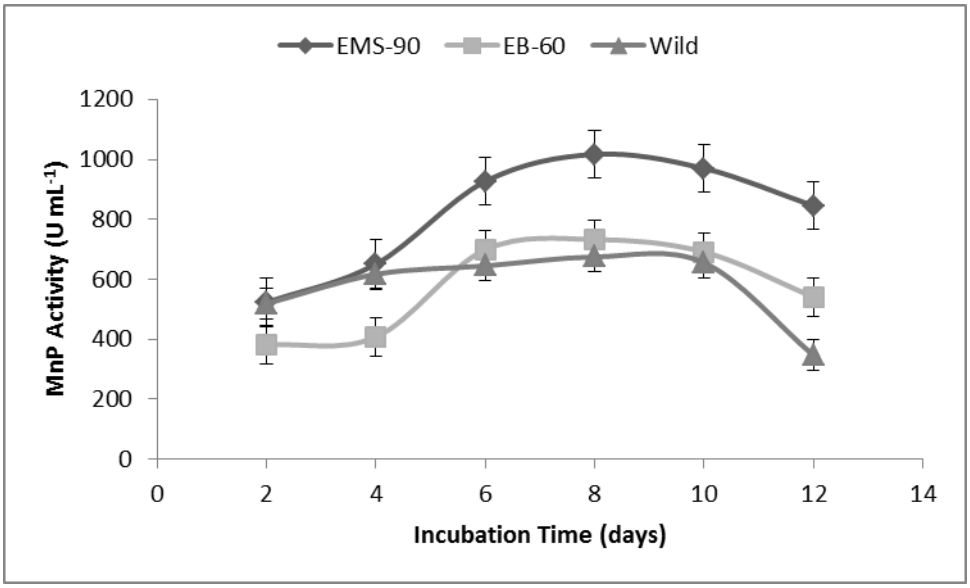

Fig. 5. Effect of incubation time on MnP production by parent and mutant strains of *T. versicolor* IBL-04

Effect of pH

Maximum enzyme activity was noted when the initial pH of the fermentation medium was kept at 5.0 (Fig. 6).

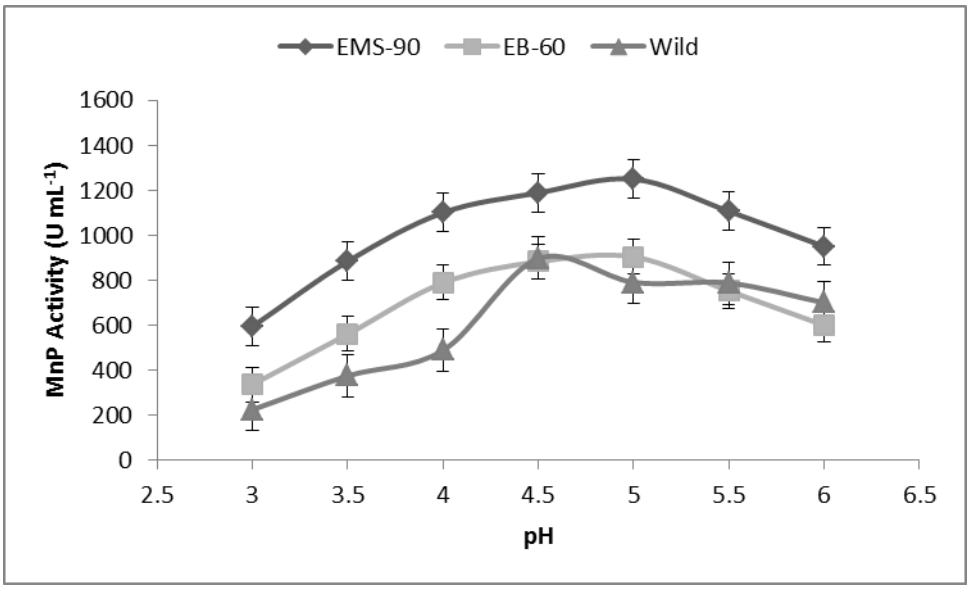

Fig. 6. Effect of pH on MnP production by parent and mutant strains of *T. versicolor* IBL-04

It was noted that an increase in initial pH enhanced MnP production by the parent and both mutant strains of *T. versicolor* IBL-04. A further increase in pH decreased the enzyme synthesis by the fungi. EMS-90 and EB-60 showed 1250 U mL^{-1} and 904 U mL^{-1} enzyme activities, respectively, but the parent strain produced the maximum enzyme concentration (897 U mL^{-1}) at pH 4.5. Optimization of pH in culture media is always important and critical for successful fermentation operations. An increase or decrease in pH can affect fungal growth and enzyme formation. Yamanaka *et al*. (2008) reported the production of ligninase by *C. hirsutus* and *T. villosa* CCB176 at pH 4.0 and 5.0,

respectively, and our results also showed maximum activity at pH 5.0 for selected mutant strains.

Effect of Temperature

Optimization of temperature was carried out by incubating the inoculated media flasks at varying temperatures. Maximum enzyme production was noted in the flasks incubated at 35 °C, and a further increase in temperature caused the enzyme activity to drop (Fig. 7). The EMS-90, EB-60, and parent strain showed enzyme activities of 1435 U mL^{-1}, 988 U mL^{-1}, and 805 U mL^{-1}, respectively. It is likely that the most important factor among all the physical variables affecting the SSF performance is the incubation temperature because higher temperature had some adverse effect on the metabolic activities of the microorganisms (Weng and Sun 2006). This might be due to the fact that high temperature has an inhibitory effect on the growth of microorganisms as well as the productivity of enzymes. Tekere *et al*. (2001) reported an optimum temperature of 35 °C to 37 °C for *Coriolus versicolor* in SSF for ligninolytic enzyme production.

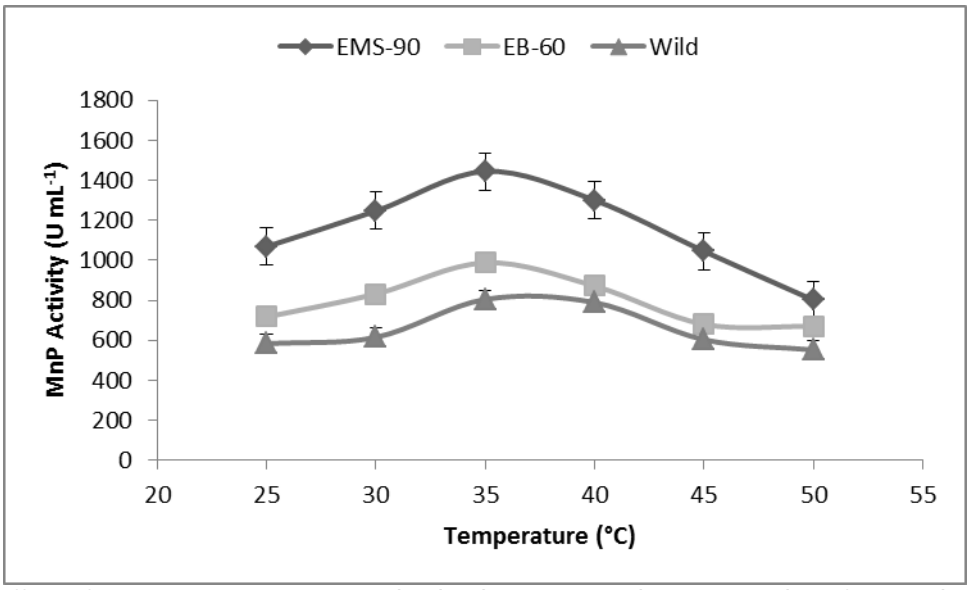

Fig. 7. Effect of temperature on MnP production by parent and mutant strains of *T. versicolor* IBL-04

Effect of Inoculum Size

Varying volumes of inoculum (1×10^7spores mL^{-1}) were used for optimization of this parameter, and its effect on MnP production was studied. It was noted that enzyme activity increased with increasing inoculum size, and at a 4-mL inoculum size, MnP activity was maximum for all strains (Fig. 8). The following maximum enzyme activities for the selected strains were obtained: EMS-90, 1407 U mL^{-1}; EB-60, 1236 U mL^{-1}; parent, 868 U mL^{-1}. The optimum inoculum level is important for the solid state fermentation process. It has been previously reported that lower inoculum levels may not be sufficient to initiate fungal growth, resulting in a longer lag phase and subsequent enzyme formation (Iqbal *et al*. 2011). On the other hand, a higher inoculum level increases spore density, causing faster depletion of available nutrients (Patel *et al*. 2009). Higher inoculum volumes also decrease the substrate:water ratio, thus decreasing

aeration in the solid substrate matrix, which ultimately inhibits fungal growth and enzyme secretion.

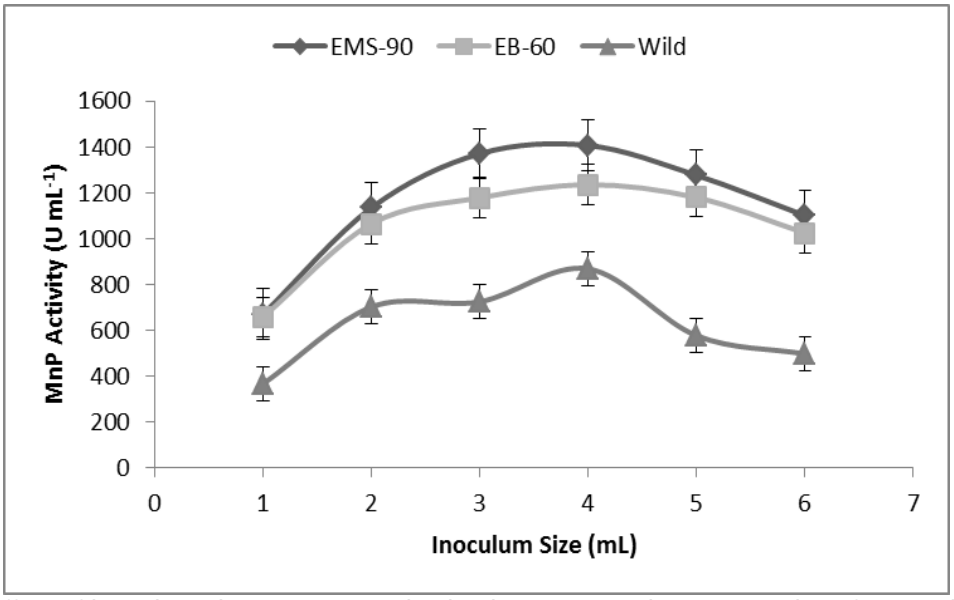

Fig. 8. Effect of inoculum size on MnP production by parent and mutant strains of *T. versicolor* IBL-04

Effect of Moisture Level

The effect of different moisture levels on MnP production by the parent and mutant strains was studied. Optimization of initial moisture content were undertaken and varied from 40% to 70%. Various volumes of Kirk's medium (2, 4, 6 mL) were used to moisten 5.0 g of substrate in triplicate flasks. The moisture content was expressed as weight of moisture (1 mL of water/medium nearly equal to 1 g) per g of substrate (% w/w). Optimum MnP production was observed with a 60% moisture level (Fig. 9).

Sugar-cane bagasse fermented at 60% (*w/w*) moisture resulted in maximum MnP activity (EMS-90, 2031 U mL^{-1}; EB-60, 1509 U mL^{-1}; parent, 1177 U mL^{-1}). All other moisture levels produced lesser enzyme activities. When moisture level is increased up to certain limits, the diffusion of enzyme, nutrients and products is increased. Moisture level is one of the critical parameters in solid state fermentation (Demirel *et al*. 2005), and optimum moisture in low level range is beneficial for SSF, as it reduces the chances of contamination. However, very low moisture contents reduce heat and mass transfer during fermentation, and higher moisture levels reduce the inter-particle space, thereby reducing oxygen penetration (Khosravi-Darani and Zoghi 2008).

Effect of C/N Ratio

An increase in enzyme production resulted from increasing the C/N ratio from 5:1 to 20:1, and MnP activity peaked in medium containing a 20:1 C/N ratio (Fig. 10). The optimum C:N ratio should be maintained to enable the fungus to carry out its metabolic activities and to synthesize nitrogen-containing compounds including the enzyme proteins. EMS-90, EB-60 and the parent strain showed 3217 U mL^{-1}, 2387 U mL^{-1} and 1340 U mL^{-1} MnP activities, respectively. At low C:N ratios, the fungus was carbon starved, and high-nitrogen conditions prevailed. In a previous study, the formation of MnP was considerably reduced by a low C:N ratio (Xiong *et al.* 2008).

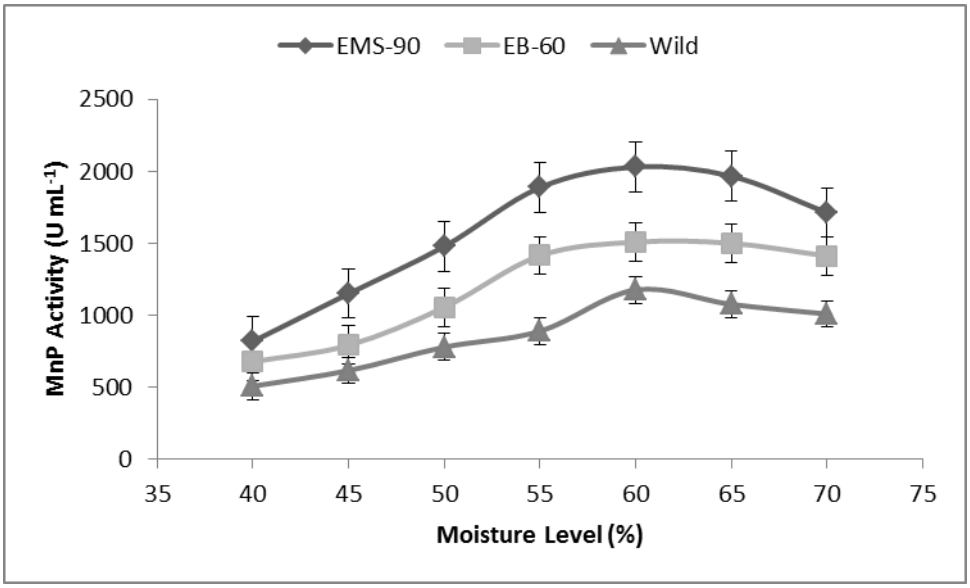

Fig. 9. Effect of moisture content on MnP production by parent and mutant strains of *T. versicolor* IBL-04

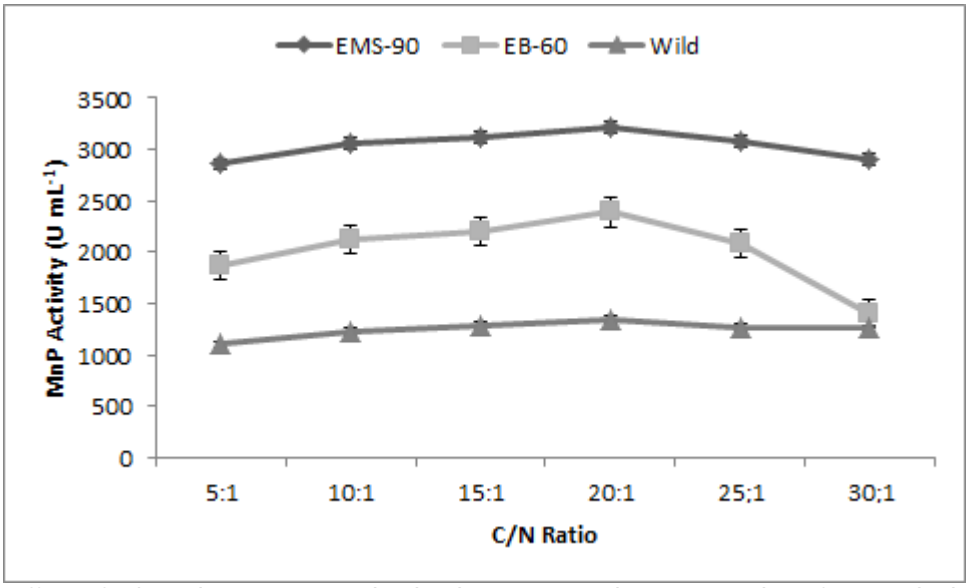

Fig. 10. Effect of C/N ratio on MnP production by parent and mutant strains of *T. versicolor* IBL-04

Effect of Mediators

Five different mediators, manganese sulfate, ABTS, oxalate, veratryl alcohol, and hydrogen peroxide were used in 1 mM concentrations to investigate their stimulatory/ inhibitory influences on MnP production. It was observed that manganese sulfate stimulated MnP production (Fig. 11). EMS-90 had an activity of 3637 U mL^{-1} and EB-60 had an activity of 2688 U mL^{-1} when manganese sulfate was used as a mediator, whereas other used mediators had little or no effect on MnP activity. Urek and Pazarlioglu (2007) also reported enhanced MnP activity by adding manganese sulfate in fermentation medium.

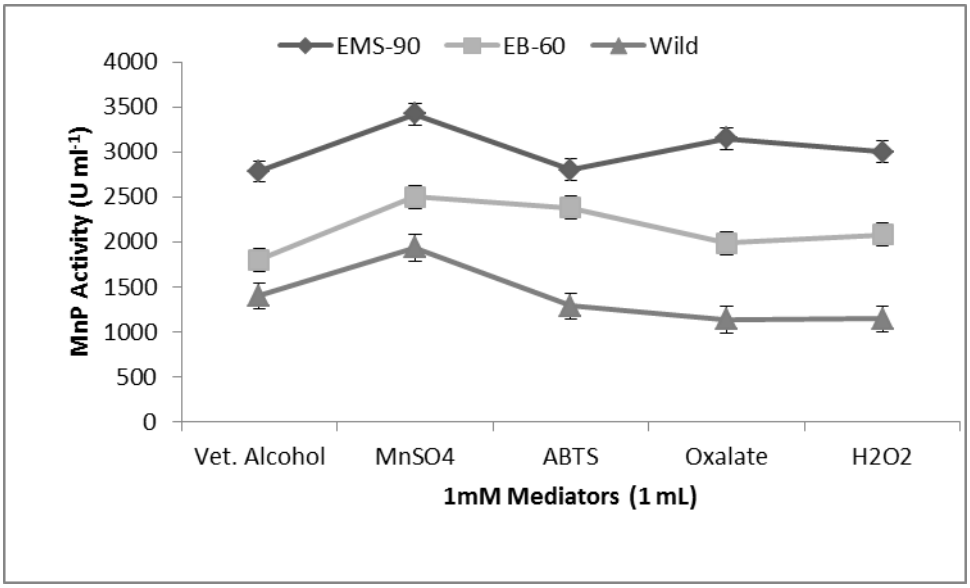

Fig. 11. Effect of mediators on MnP production by parent and mutant strains of *T. versicolor* IBL-04

Several studies have shown that ligninolytic enzyme production could be enhanced several folds by the addition of mediators (Gnanamani *et al*. 2006; Minussi *et al*. 2007). Manganese is a critical nutritional variable in the production of ligninases, including LiP and MnP by *Phanerochaete chrysosporium* **(**Pascal *et al*. 1991). MnP production by *Lentinula edodes* on corncob solid state cultures was also influenced by mediators (Boer *et al*. 2006).

CONCLUSIONS

The chemical mutagenesis of *T. versicolor* IBL-04 resulted in the development of hyper-producing mutants for MnP production in solid state fermentation (SSF). An increase of 3.2-fold in enzyme production was achieved through chemical mutagenesis in comparison to the parent strain of *T. versicolor* IBL-04. The MnP production by the selected mutants (EMS-90 and EB-60) could be enhanced further by optimizing physical and nutritional factors. High MnP yield from mutant strains suggests the potential of chemical mutagenesis for the development of efficient fungal strains for commercial-scale production and industrial applications.

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