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Full Length Article

Purification and Characterization of Novel Manganese Peroxidase from *Schizophyllum commune* IBL-06

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Abstract

A novel manganese peroxidase (MnP) was isolated from an indigenous fungal species *Schizophyllum commune* strain IBL-06 that was grown in pre-optimized solid state culture using banana stalk as substrate. The MnP was purified by $(NH_4)_2SO_4$ fractionation, dialysis and Sephadex G-100 gel filtration chromatography to achieve 1.8 fold purification with 22% enzyme yield and 506 U/mg specific activity. A homogenous single band of 40 kDa for purified MnP was obtained after gel documentation of Native-PAGE that was further confirmed on SDS-PAGE. Characterization of MnP revealed that the pH and temperature optima of *S. commune* IBL-06 MnP were 5 and 40°C, respectively. *S. commune* MnP showed high substrate ($MnSO_4$) affinity and catalytic efficiency with corresponding kinetic constants K_m and V_{max} values of 0.4 mM and 410 mM/min, respectively. The purified enzyme was activated by $CuSO_4$ and $MnSO_4$ and inhibited by $CaCl_2$, EDTA, TEMED, β-Marcaptoethanol, $AgNO_3$ and $Pb(NO_3)_2$ (most inhibitory). © 2013 Friends Science Publishers

Keywords: *S. commune* IBL-06; MnP; Purification; Gel filtration; SDS-PAGE; Characterization

Introduction

Ligninolytic system of White rot fungi (WRF) comprising manganese peroxidase (MnP), lignin peroxidase (LiP) and laccase is endowed with potential capabilities to depolymerize/degrade lignin and recalcitrant environmental pollutants such as textile dyes, poly aromatic hydro carbons, poly chloro-phenols and poly chlorinated bi-phenyls that pose serious health hazards to animals and human beings (Revankar and Lele, 2006; Asgher *et al*., 2008). The occurrence of MnP and laccase is higher than LiP and both enzymes have extraordinary potential to oxidize the range of different phenolic and non-phenolic complex compounds and other toxic compounds under certain conditions (Wesenberg *et al.,* 2003; Katia *et al.,* 2005; Stoilova *et al.,* 2010; Asgher *et al.,* 2012). MnP is a heme containing extracellular glycoprotein synthesized during secondary metabolism by litter decomposing basidiomycetes and some ascomycetes. During enzymatic action MnP catalyzes the H_2O_2 dependent oxidation of Mn^{2+} to a reactive Mn^{3+} (Asgher and Iqbal, 2011).

During recent years, manganese peroxidase (MnP) and laccases have been applied to numerous processes such as in biopulping, biobleaching, bioremediation, deliginification, oxidation of organic pollutants, stabilization of fruit juices, biosensors development, biofuels cells, textile biofinishing, beverage processing, animal feed stuffs, cosmetics, detergent manufacturing and transformation of antibiotics and steroids (Miele *et al*., 2010; Uzan *et al*., 2010; Aracri and Vidal, 2011; Asgher and Iqbal, 2011; Asgher *et al*., 2012; Fillat *et al*., 2012).

Banana is one of the most consumed fruits in the world and Pakistan as well and produces abundant magnitude of lignocellulosic banana wastes like fruit peel, pseudostem and stalks. SSF using banana stalks is an attractive option for enzyme production from fungal cultivations (Reddy *et al*., 2003). Keeping in view the extensive industrial applications of MnP, the present study was performed with an objective to purify and characterize the extracellular MnP from an indigenous WRF strain of *S. commune* IBL-06, which secretes MnP in high titters as compared to previously reported different strains of *Schyzophyllum Sp* under optimum physical and nutritional conditions.

Materials and Methods

Chemicals and Lignocellulosic Substrate

Coomassie Brilliant Blue G-250, sodium dodecylsulphate, Sephadex G100, N, N, N′, N′-tetra-methylethylenediamine, and ß-mercaptoethanol were from Fluka-Sigma-Aldrich (USA) and Standard Protein markers were obtained from Fermentas, U.K. Locally available banana stalk was used as growth promoting substrate. The substrate was washed, oven dried at 60°C, ground into 40 mm mesh size and finally stored in polyethylene bags to avoid free moisture content until further use.

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MnP Producing Organism and its Maintenance

A pure culture of WRF strain *S. commune* was previously available in our lab (Industrial Bio-technology Lab, Department of Chemistry and Biochemistry, University of Agriculture Faisalabad, Pakistan) and used for the present investigations. The strain was multiplied on potato dextrose agar (PDA) slants having pH 4.5 followed by an incubation at 35°C for five consecutive days and stored at 4°C in refrigerator and revived periodically for whole experiment.

Fungal Spore Inoculum

For the preparation of inoculum, a loopful of *S. commune* IBL-06 from PDA slant was transferred to the inoculum medium of pH 4.5 having ingredients composition in g/L: glucose 2; $MgSO_4 \cdot 7H_2O$ 0.05; $CaCl_2 \cdot 2H_2O$, 0.1; Ammonium chloride, 0.12 and thiamine, 0.001 (Asgher *et al*., 2006) and incubated in a temperature controlled (35°C) shaking incubator at 150 rpm for 5 days to get a homogeneous inoculum suspension.

MnP Production in Pre-optimized Solid State Fermentation Process

Triplicate flasks contained 5 gm banana stalk, 60% moistened with modified Krik's salt nutrient medium (Tien and Kirk, 1988), were autoclaved and then inoculated with 3 mL of freshly prepared fungal spore suspension and incubated undisturbed at 35°C for five days in a still culture incubator (MIR-254, Sanyo, Japan).

Harvesting and MnP Extraction

After five days of bio-processing, up to 100 mL of sodium malonate buffer (50 mM) was added to the fermented mash and all of the experimental flasks were shaken (120 rpm) for half an hour and filtered through filter paper (125 mm) to extract the extracellular enzymes. To remove fungal pellets, the filtrates were centrifuged (5000 g, for 15 min at 4°C) and clear supernatants were used as crude enzyme extracts for purification and characterization studies.

Determination of MnP Activity and Protein Contents

Manganese peroxidase activity was measured spectrophotometrically with UV/Vis Spectrophotomter (Model: T60, PG Instruments, UK) using $MnSO_4$ as assay substrate by adopting the methodology as described earlier (Asgher and Iqbal, 2011). To determine the protein contents of the fermented samples, Bradford micro assay was adopted using bovine serum albumin as standard reagent (Bradford, 1976).

Fractional Precipitation for MnP Purification

Crude MnP extract was centrifuged and the collected supernatant was concentrated by freeze drying. The concentrated active fractions were placed in an ice bath followed by the continuous addition of ammonium sulfate crystals to attain 30% saturation and kept overnight at 4°C. The fraction saturated with 30% ammonium sulfate was centrifuged at 5,000×g for 30 min at 4°C. Finally to attain 55% ammonium sulfate saturation more crystals of ammonium sulfate were added in the collected supernatant and kept at 4°C for 6 h, followed by centrifugation as described above. The 50 mM malonate buffer was used to dissolve the pellets and it was dialyzed against the same buffer after sealing securely in a dialysis bag to remove extra salt and the dialyzate was finally freeze dried. Total proteins and MnP activity were determined before and after dialysis as described previously.

Gel Filtration Chromatography

The active MnP fractions were loaded on the Sephadex-G-100 column (2×25 cm) for further purification. The 600 µL/run of sample in 50 mM malonate buffer of pH 4.5 was used as elution buffer with flow rate of 0.3 mL/min. Up to 20 different active fractions (1 mL each) were collected and monitored for MnP activity.

Estimation of Molecular Weight

To confirm the purification of MnP, the purified enzyme was run on Native as well as sodiem dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) following the method of Laemmli (1970), using broad range low molecular weight markers (21-116 kDa).

Characterization of Purified MnP

The purified active MnP fraction was subjected to characterization through kinetic studies by studying the effect of pH, temperature and substrate concentraion on MnP activity and stability:

Effect of pH

MnP was assayed at 25ºC using buffers of varying pH ranging from 3-9 (50 mM sodium tartrate buffer of pH 2 and 3, 50 mM malonate buffer and 50 mM succinate buffer of pH 4 and 5, 50 mM citrate buffer and 50 mM sodium phosphate buffer of pH 6 and 7 and 50 mM sodium phosphate buffer and 50 mM potassium carbonate buffer of pH 8 and 9). For stability studies the enzyme was incubated at varying pH for 2 h before routine assay protocol.

Effect of Temperature

Effect of varying temperature in the range of 25-70ºC on purified MnP activity and stability was investigated (Asgher and Iqbal, 2011). The enzyme was incubated for 30 min at different temperatures without substrate before carrying out MnP assay. For stability studies MnP was incubated for 2 h at different temperatures prior to normal activity assay.

Determination of Kinetic Constants K_m and V_{max}

The kinetic constants K_m and V_{max} were determined by investigating the effect of different concentrations of $MnSO_4$ as assay substrate in 1-5 mM concentration range. The data was manipulated to construct the reciprocal plot using Lineweaver-Burk plot transformation of the Michaelis-Menton kinetic rate equation.

Effect of Activators/Inhibitors

The effects of various organic compounds such as Tetramethylethylenediamine (TEMED), Marcaptoethanol, Ethylenediaminetetraacetic acid (EDTA) and metal ions including Pb^{2+}, Ca^{2+}, Cl^{2-}, Mn^{2+}, Cu^{2+} on MnP activity were studied in the concentration of 1-5 mM.

Results and Discussion

MnP Purification

MnP from *S. commune* IBL-06 produced on banana stalks solid state culture under some previously optimized fermentation conditions (Irshad *et al.*, 2011) was salted out at 55% saturation with $(NH_4)_2SO_4$ to 1.4 fold purification with specific activity of 394 U/mg. After Sephadex G-100 filtration chromatography (Fig. 1), the specific activity of MnP increased to 506 U/mg with 1.8 fold purification and 22% activity yield (Table 1). Previously, MnP from *Phanerochaete sp* was salted out at 65% $(NH_4)_2SO_4$ saturation with 2.68 purification fold and 5.56% overall yield (Rajan *et al.*, 2010). Hofrichter *et al.* (1999) separated the pooled ligninolytic activities by sephadex column and MnP was eluted out as a single peak with tris buffer of pH 8.0 with 0.1M NaCl, showing that this enzyme was less anionic. Fractionation of MnP from *P. chrysosporium* by DEAE Sepharose, followed by ion-exchange chromatography and UltragelAcA54 gel filtration chromatography resulted in 23.08% activity yield with 5.8 fold of purification (Ürek and Pazarlioğlu, 2004).

SDS-PAGE for MnP

A homogenous single band of of 40 kDa for purified *S. Commune* IBL-06 MnP was obtained after gel documentation of Native-PAGE that was further confirmed on the SDS-PAGE (Fig. 2A and B), suggesting that the enzyme was a single polypeptide protein. MnP family peroxidases have previously been reported to be the extracellular glycoproteins containing heme prosthetic groups with molecular weights varying from 32-75 kDa (Ürek and Pazarlioğlu, 2004; Baborová *et al*., 2006). The molar masses for MnPs from *B. Pumilus* and *Paenibacillus sp* on SDS-PAGE were 25 and 40 kDa, respectively (de Oliveira *et al*., 2009), whereas MnP from *Irpex lacteus* was a single polypeptide of 53.2 kDa (Shin *et al*., 2005). In our previous study (Asgher and Iqbal, 2011), MnP from *T. versicolor* IBL-04 was found to be a homogenous monomeric protein of 43 kDa on SDS-PAGE. Cheng *et al*. (2007) also reported 48.7 kDa molar mass for MnP from *Schizophyllum sp.* F17.

Characterization of MnP

Effect of pH on MnP activity and stability: The pH-activity profile for MnP (Fig. 3) showed that activity peaked (877 U/mL) at pH 5 and a further increase in pH caused gradual deactivation of the enzyme. MnP was fairly stable between pH ranges 4-6 when incubated for 2 h. In previous studies pH optima for MnP from different WRF have been reported in the range of 4-7 (Bermek *et al.*, 2004; Ürek and Pazarlioğlu, 2004; Hakala *et al.*, 2005; Baborová *et al.*, 2006; Asgher *et al.*, 2008; de Oliveira *et al.*, 2009). MnP produced by *Lentinula edodes* in SSF of corncobs was optimally active at pH 4.5 (Boer *et al.*, 2006). Another MnP isolated from *S. commune* IBL-06 grown on banana stalks solid state culture was optimally active at pH 5.

Effect of temperature on MnP activity and stability: Temperature verses MnP activity curve (Fig. 4) showed an initial increase in MnP activity with temperature but at higher temperatures, the enzyme showed a rapid activity loss due to heat denaturation. The optimum temperature for $MnSO_4$ oxidation was 60°C. The enzyme was stable upto 50°C and it retained almost 90% of its activity after 1 h incubation. However, MnP lost 45% of its activity when incubated at 60°C for 2 h. Most of the earlier reported MnPs have been reprted to lose their activities at temperatures around 60°C. MnPs from different WRF have been reported to have optimum temperatures of 40-60°C (Bermek *et al.*, 2004; Ürek and Pazarlioğlu, 2004; Hakala *et al.*, 2005; Baborová *et al.*, 2006; Asgher *et al.*, 2008; de Oliveira *et al.*, 2009). According to the Shin *et al.* (2005) MnP from *Irpex lacteus* was stable in the range of 30 to 40°C, whereas MnP from another WRF strain, *Rhizoctonia* sp. SYBC-M3 was stable at 55°C (Cai *et al.*, 2010). The MnP2 isozyme from *Lentinula edodes* produced in SSF of corn cobs had 40°C optimum temperature (Boer *et al.*, 2006).

Determination of kinetic constants K_m and V_{max}: Effect of varying concentrations of $MnSO_4$ on the activity of MnP was studied using reciprocal plot of 1/S Vs 1/V (Fig. 5) to determine the Michaelis–Menten constants K_m and V_{max}. The K_m and V_{max} values for the enzyme were 0.4 mM and 410 mM/min, indicating a very high affinity and catalytic efficiency of *S. commune* IBL-06 MnP as compared to most of the previously reported MnPs from various WRF. The K_M value of MnP2 for $MnSO_4$ was 22.2×10^{-3} mM (Boer *et al*. 2006), while the MnP from *Bjerkandera sp.* strain BOS55 expressed the K_m value of 51 µM and turn over number of 59/s (Mester *et al*., 1998). The *Km* values of MnP for H_2O_2 and 2.6-dimetoxyphenol were 71.4 and 28.57 µM, respectively at pH of 4.5 (Ürek and Pazarlioğlu, 2004).

Table 1: Purification summary for MnP produced by *S. commune* IBL-06

Purification Steps	Total Volume (mL)	Total Enzyme Activity (IU)	Total Protein Content (mg)	Specific Activity (U/mg)	Purification fold	% Yield
Crude MnP	500	1857000	6628	280	1	100
$(NH_4)_2SO_4$ Precipitation	40	1158160	2934	394	1.4	62
Dialysis	40	948370	2103	450	1.6	51
Sephadex-G-100	12	418546	826	506	1.8	22

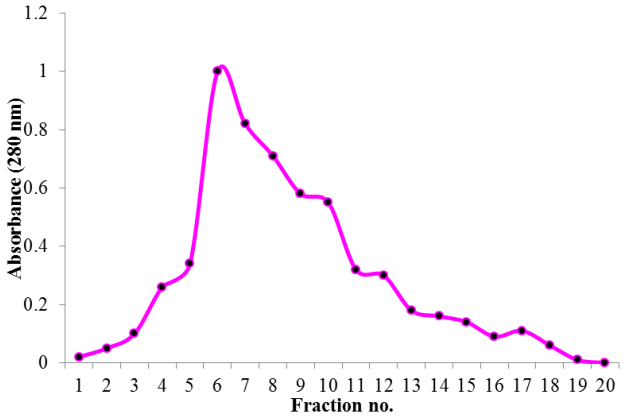
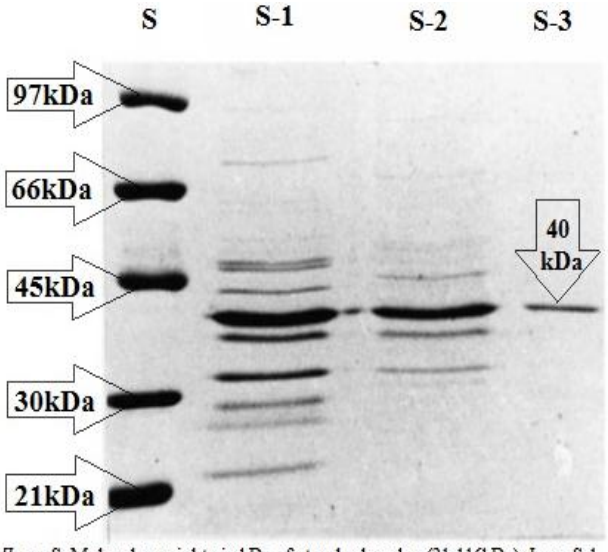
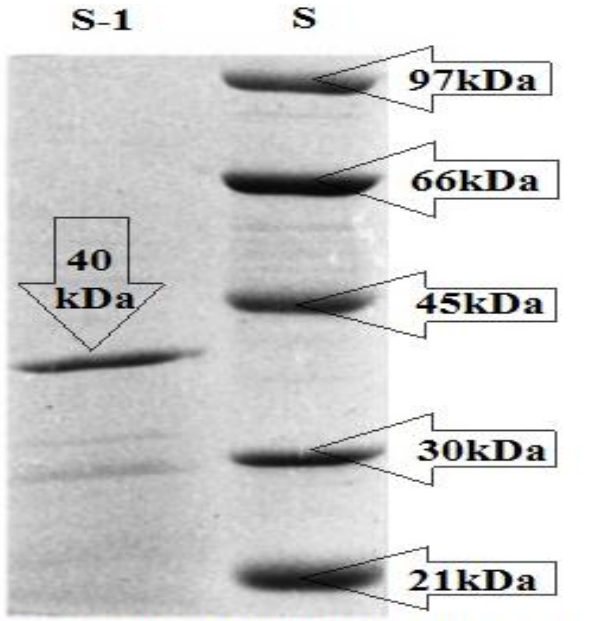


Fig. 1: Gel filtration chromatography of MnP produced by *S. commune* IBL-06



[Lane S, Molecular weights in kDa of standard marker (21-116kDa); Lane S-1, Crude MnP extract; Lane S-2, Partial purified MnP after $(NH_4)_2SO_4$ precipitation; Lane S-3, Purified MnP after Gel filtration chromatography]

Fig. 2A: Native-PAGE for MnP produced by *S. commune* IBL-06



[Lane S, Molecular weights in KdA of standard marker (21-116kDa); Lane S-1, Purified MnP after Gel filtration chromatography]

Fig. 2B: SDS-PAGE for MnP produced by *S. commune* IBL-06

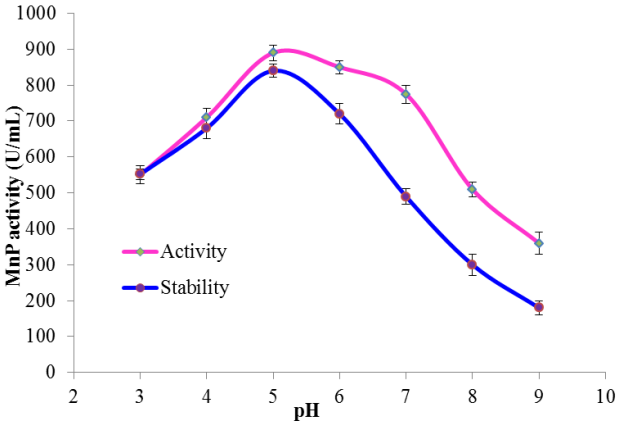


Fig. 3: Effect of pH on activity and stability of MnP from *S. commune* IBL-06

Effect of Activators and Inhibitors

The effects of varying concentrations of different activators/inhibitors on MnP activity were investigated using $MnSO_4$ as substrate. Cu^{2+} and Mn^{2+} ions (1-4 mM) were found to activate *S. commune* MnP, whereas all other metal ions as well as organic compounds were found inhibitory; $AgNO_3$ being the strongest inhibitor (Fig. 6). *P. chrysosporium* MnP activity was inhibited by NaN_3, β-mercaptoethanol and dithreitol (Ürek and Pazarlioğlu, 2004), whereas the activity could be enhanced in the presence of co-oxidants such as glutathione, un-saturated fatty acids and Tween 80 (Hofrichter *et al.*, 1999; Ürek and

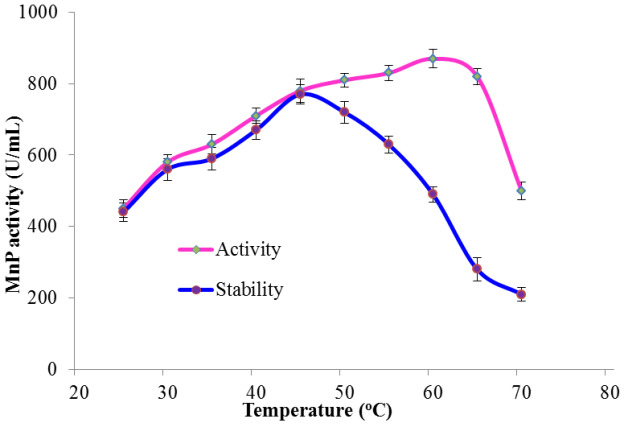


Fig. 4: Effect of temperature on activity and stability of MnP from *S. commune* IBL-06

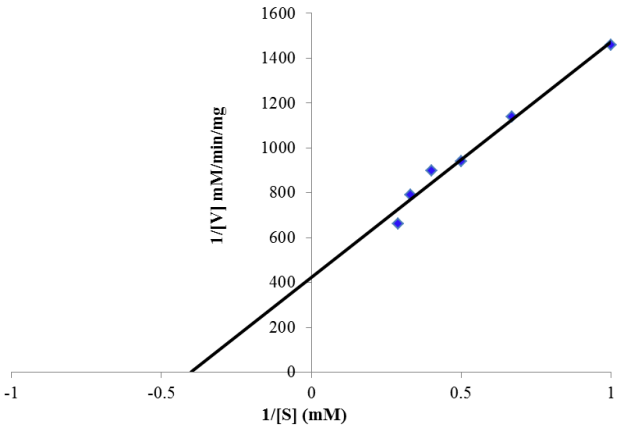


Fig. 5: Reciprocal plot of 1/[S] Vs 1/[V] for determination of K_M and V_{max} for MnP produced by *S. commune* IBL-06

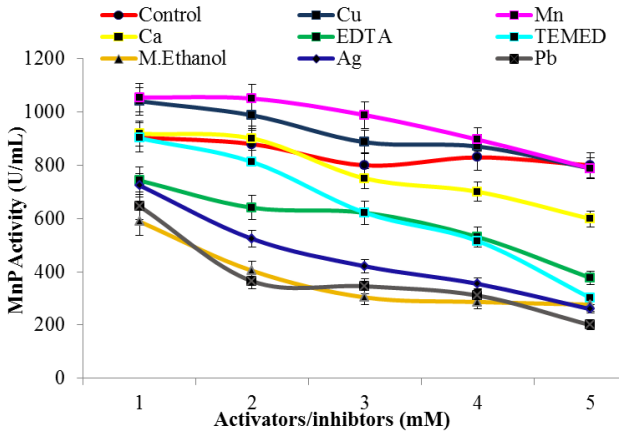


Fig. 6: Effect of activators/inhibitors on purified MnP produced by *S. commune* IBL-06

Pazarlioğlu, 2004). The MnP from *Trichophyton rubrum* LSK-27 was completely inhibited by Hg^{2+}, while Fe^{3+}, Ca^{2+} and Ni^{2+} did not cause inhibition in the enzyme activity (Bermek *et al.*, 2004; Boer *et al.*, 2006; Cheng *et al.*, 2007). In a previous study (Asgher and Iqbal, 2011) the inhibitory effects of metal ions like Ag^{+} and Fe^{2+} MnP from *T. versicolor* IBL-04 have also been reported.

Conclusion

The purified S. commune IBL-06 MnP showed novel biochemical features, including higher stability against varying temperatures and pH, high substrate affinity and catalytic efficiency as compare to previously reported MnPs from different WRF. The high levels of MnP secreted by this indigenous strain as well as its high thermo-stability suggest that it could be a useful tool for biotechnological applications. Immobilization of MnP for improving its thermo-stability characteristics is the focus of future research.

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