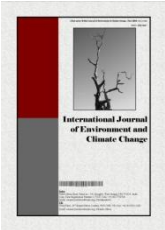

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Decolorisation of Azo Dye Congo Red (CR) by *Termitomyces* sp. Biomass

Kavitha Mary Jackson[1*] and Velu Gomathi[1]

[1]Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India.

Authors' contributions

This work was carried out in collaboration between both authors. Author KMJ designed the study, performed the experiments and statistical analysis and wrote the first draft of the manuscript. Author VG guided the entire study and corrected the manuscript. Both authors read and approved the final manuscript.

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ABSTRACT

Aims: A study was conducted to evaluate decoloration of azo dye, Congo Red (CR) using fungal hyphal mat of beneficial bacidiomycete *Termitomyces* sp. TMS7 (MW694830) as bio sorbent material.
Study design: Completely randomized block design (CRD).
Place and duration of study: Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India, between September 2019 and January 2020.
Methodology: Isolation of white rot fungus from basidiocarb was done and screened based on their ligninolytic enzyme activity and Isolate TMS 7 was selected as best isolate and identified through ITS 1 and ITS 4 primers. Efficiency of fungal biomass to decolorize Congo red was assessed and per cent decoloration and kinetics were calculated.
Results: Twelve fungal isolates were obtained and Isolate TMS 7 was selected as best isolate based on enzymatic activity. TMS 7 was identified as *Termitomyces* sp. using ITS 1 and ITS 4 primer. Ligninolytic enzymes i.e. cellulase (9.97 µ mol of glucose released/min/mg protein), and xylanase (9.55 µ mol of xylose released/min/mg protein) were quantified from the crude fungal extract of TMS 7, which was higher than standard (*Termitomyces albuminosus* -MTCC 1366). Decolorisation efficiency of termitomyces fungal biomass (1 g/100 ml) against different concentration of congo red dye (50-250 mg/L) was assessed. About 100 % (99.9) degradation was recorded in the minimum dye concentration of 50 mg/L within 3 days and 8 % decoloration was

**Corresponding author: E-mail: kavitha_jackson@yahoo.co.in;*

achieved at the highest dye concentration (250 mg/L) within 5 days.
Conclusion: Possible mechanism of degradation is the presence of lignolytic enzyme especially cellulase, xylanase in the culture filtrate and bio sorption of degraded product by the fungal cell wall components viz., chitin, glucan other complex polymers.

Keywords: Termitomyces sp.; fungal mat; biosorbent; decoloration.

1. INTRODUCTION

Textile industries hold a major share in India's GDP (Gross Domestic Price) and export. Dyeing of fabric/fibre is a quintessential step in textile industry. Different types of synthetic dyes were used along with some mordant. Dyes were not fixed 100% in fibres and half of the proportion were mixed with effluent and disposed into environment. Due to the toxicity of their decomposition products to aquatic organisms, dyes have to be removed from effluents before release [1]. Therefore various environmental protection acts were enforced worldwide, which obliges the textile industries to treat the effluents before disposal to environment. In India, Tirupur district of Tamil Nadu state has 760 textile dyeing units established out of which 430 units are in operation. These units have installed Common Effluent Treatment Plants (CETPs) consisting of physical, chemical and biological treatment units. Some of the units have installed Individual Effluent Treatment Plants (IETPs). The treated effluent was finally discharged into the river. However CETPs and IETPs failed to meet discharge standards of TDS [Total Dissolved Solids] and chlorides and thereby significantly affected the river water quality [2].

Various physical/chemical methods have been used for the removal of dyes from wastewater such as adsorption, precipitation, flocculation, flotation, coagulation, and electrochemical destruction methods [3]. However these methods are not economically feasible and produce large amounts of sludge [4,5].

Adsorption is the most common dye removal method, because it does not produce dangerous byproducts, and some of the adsorbents can be easily regenerated. Recently, various adsorbents such as zeolite, [6] multiwall carbon nanotubes,[7] graphene oxide,[8] and calcined ZnMgAl hydrotalcite [9] have been utilized for the dye Methyl Orange removal. Similarly Modified Linde-type A zeolite (LTA) was prepared from coal fly ash (CFA) and used as an adsorbent to remove acidic dye (Acid red 66, AR66) from its aqueous solution [10].

Biological treatment of textile industry effluents using microbial biodegradation of dyes is a better alternative [11]. This method is more economical and leads to less accumulation of relatively harmless sludge [12]. Bio sorption process has attracted a great interest and seems a good alternative for the removal of dyes and other pollutants from wastewaters [13]. It can be defined as sequestering of organic or inorganic compounds by alive or dead biomasses or their derivatives; the biomass can consist of bacteria [14], fungi [13], yeasts [14], algae [15], seaweeds and even industrial or agricultural wastes [16].

The most efficient microorganisms to break down colored pollutants so far reported are white-rot fungi [17,18]. These comprise mostly basidiomycetous fungi, which are capable of extensive aerobic lignin degradation and mineralization. While using fungal biomass for remediation, inactivated/dead fungal biomass is mostly used as a biomaterial for bio sorption process as it has no risk of contamination during bio sorption process [19,20].

Azo dyes are the largest group of dyes, constituting 60-70% of all organic dyes produced globally [21] and they cause potential threat to environment by reducing the transparency of water bodies. They are harmful to ecosystems if discharged to the water system. Congo red is a widely used azo dye in the textile dyeing, due its high solubility in water. They contain sulfonic groups and diazo (N=N) groups, hence considered as highly resistant to any sort of degradation. Various fungal sp. such as *Aspergillus niger* [22] *Phanerochaete chrysosporium* [23], *Caldariomyces fumago* [24], *Curvularia* sp. [25] and *Trametes versicolor* [26] were reported earlier as Congo red degrading fungi. In the current investigation *Termitomyces* sp. used as biosorbent material to remediate Congo red dye from the aqueous solution. As it is an edible mushroom, toxicity concerns won't raise, if the fungus is released into environment directly.

2. MATERIAL AND METHODS

2.1 Fungal Strains and Growth Conditions

Microorganisms used in this study *Termitomyces* sp. (GenBank accession No. MW694830) was isolated from fruiting body (basidiocarp) of white fungus collected from Western Ghats of Tamil Nadu, India during North east monsoon (September to October, 2019). Twelve fungal isolates were obtained and screened based on their enzymatic activity. Standard culture *Termitomyces albuminosus* (MTCC 1366) was obtained from Indian Institute of Microbial Technology, Chandigarh. The cultures were maintained in Potato dextrose Agar slants at 4°C and sub-cultured periodically.

2.2 Screening of Termitomyces Isolates Based on Enzymatic Activity (Plate Assay)

Termitomyces isolates were screened based on the production of Glycosyl Hydrolase enzymes i.e. cellulase and xylanase on selective minimal medium amended with 1% carboxymethyl cellulose (CMC) and xylan respectively. Pure cultures were spot inoculated on 1% CMC and xylan agar plates and incubated at 37ºC. After 3-5 days, the plates were flooded with 1% Congo-Red solution for 15-20 min, and then de-stained with 1M NaCl solution for 15-20 min [27]. Qualitative scoring were given based on the clearance of substrate i.e. CMC and xylan.

2.3 Quantitative Assay of Ligninolytic Enzymes

Screened fungal isolate was subjected to quantitative estimation of lignolytic enzymes following standard protocol i.e., cellulase [28] and xylanase [29]. Fungal isolates were grown in Czapek-dox broth ($MgSO_4.7H_2O$ - 0.5g/L;$FeSO_4$ - 0.01g/L;KCl - 0.5g/L;KH_2PO_4 - 1g/L ;$NaNO_3$- 3g/L;pH – 6.0-6.5) with 1% of respective carbon source i.e., carboxy methyl cellulose (CMC) for cellulase and xylan for xylanase.

2.4 Estimation of Decolourization of Dye Congo Red

The fungal mat (1 g) was added to 100 ml of aqueous solution containing the congo red dye at different concentrations (50, 100, 150, 200, 250, 500 mg/L) and the decolourization of the dye was measured spectrophotometerically at 566 nm [Spectramax® i3X (Molecular devices) Multi-Mode Microplate Reader (USA)] and optical density (OD) were recorded. For the effect of initial concentration of dyes, the samples were analysed after 5 days of incubation against a similarly treated medium blank. For decolorisation kinetics, the samples were drawn at every 24 h interval and analyzed using the same procedure. The respective dye samples were used as standard.

3. RESULTS AND DISCUSSION

3.1 Collection and Isolation of *Termitomyces* sp. from Western Ghats of Tamil Nadu

Termitomyces sp. an edible mushroom produces larger quantity of ligninolytic enzymes especially cellobiose dehydrogenase, which is highly applicable in terms of textile effluent treatment were taken for this study. *Termitomyces* sp. reported as reactive blue dye degrader in earlier studies [30]. Their complex polymeric cell wall materials (i.e., pectin, chitin, glucan etc.) might involve in the action of bio sorption. In India occurrence of this fungus mostly recorded in Western Ghats of India spawning Kerala, Karnataka, Tamil Nadu and Goa. *Termitomyces microcarpus, T. clypeatus* are mostly occurred in Tamil Nadu. In the current study, a total of twelve fungal isolates were isolated from termite mound soil as well as fruiting body of termitomyces collected from Western Ghats of Tamil Nadu, India during September to November (Fig 1a& 1b). Earlier Karun and Sridhar [31] reported occurrence of *Termitomyces* sp. from Kodagu District and Dakshina Kannada District, Karnataka during southwest monsoon and post-monsoon seasons (June–November).

3.2 Screening of Termitomyces Isolates Based on Enzymatic Activity (Plate Assay)

Enzymes, primarily the lignin degrading enzymes, have been successfully used for decoloration of a variety of dyes [32,33]. A study conducted by Bashir et al. [34] revealed that *Termitomyces* sp. OE147 produces a wide array of oxidoreducatase (Cellobiose dehydrogenases (CDH), laccases and some uncharacterized oxidoreductases) in the culture fluid when

cultivated on cellulose. In the present investigation fungal isolates were screened based on their ligninolytic enzyme i.e. cellulsse, xylanase production. Enzyme production was tested qualitatively in plate assay and scoring was given based on the clearance of substrate (cellulose and xylan). Isolate TMS 7 shows higher cellulase and xylanase production followed by TMS 11 compared to other isolates and standard (*Termitomyces albuminosus* - MTCC 1366) (Table 1).

Table 1. Qualitative assay of ligninolytic enzymes produced by Termitomyces isolates

Isolates	Clearing index	
	Cellulase	Xylanase
TMS 1	++	+
TMS 2	+	+
TMS 3	+	-
TMS 4	+	+
TMS 5	+	+
TMS 6	++	+
TMS 7	+++	++
TMS 8	++	+
TMS 9	+	-
TMS10	++	-
TMS 11	+++	+
TMS 12	++	++
Standard	++	++

High (+++), Medium (++), Low (+), Nil (-)

3.3 Quantitative Estimation of Lignolytic Enzymes of Fungal Isolate TMS 7

Ligninolytic enzyme activity of TMS 7 (*Termitomyces* sp.) was assayed using standard protocols. Cellulase enzyme activity was maximum (9.97 µ mol of glucose released/min/mg protein) during 11th day after incubation, which is higher than standard (9.8 µ mol of glucose released/min/mg protein). Similarly xylanase activity recorded maximum value at 11th day after inoculation (9.55 µ mol of xylose released/min/mg protein) and decreased afterwards. This might be due to the decline phase of fungal growth. Both isolate and standard recorded similar trend, in these enzymatic estimation (Table 2). Study of Zeleke et al. [35] reveals that extracts of termite comb (Macrotermitinae) showed strong xylanase activity (8.28U g^{-1}) with no cellulose activity. Gomathi et al. [36] recorded a maximum cellulase and xylanase activity of 10.27 µmol of glucose released/min/mg protein and 9.87µmol of xylose released/ min/ mg protein respectively at 12th day after inoculation. Yang et al. [37] recorded β-Glucosidase activity of *Termitomyces* ranges from 1.025 to 1.516 (U/L).

3.4 Molecular Confirmation of Fungal Isolate by ITS1 and ITS 4

Fungal isolate (TMS 7) was identified using ITS 1 and ITS 4 primers and confirmed as *Termitomyces* sp. Nucleotide sequences were submitted in NCBI [National centre for Biotechnology Information] (GenBank accession No. MW694830). Earlier *Termitomyces* sp. isolated from Theerthahalli forest area of Western ghats, Shimoga district, Karnataka during August and identified (98% similarity) using internal transcribed spacer (ITS) region of ribosomal DNA sequences [38].

3.5 Effect of *Termitomyces* sp. on the Decolorisation of Congo Red

Biomass plays an important role in the decolorisation of the dyes, since it enhances the degradation of the dyes through increasing the activity of the enzyme. Cell biomass has been suggested as a promising way to remove the color through bio sorption [24]. In the present study Congo red was tested for 5 days to determine the ability of fungus *Termitomyces* sp. to decolorise the dye. One gram of fungal hyphal mat was taken and incubated simultaneously in aqueous solution containing different concentration of congo red (50-250 mg/L). The percentage decolorisation decreased with increase in dye concentration (Fig. 2). Maximum decoloration (100%) recorded in lowest dye concentration (50 mg/L) and minimum decoloration (8 %) recorded in higher dye concentration (250 mg/L). Results of the present investigation imply that biomass requirement for decolorisation increases with increased concentration of dye.

Chakraborty et al. [39] recorded decolorisation of congo red dye by a *Alternaria alternata* CMERI F6 and the decolorisation rate was maximal (20.21 mg/L h) at 25 °C, pH 5, 150 rpm and 800 mg/L dye, giving 78% final decolorisation efficiency. Wang et al. [40] reported decolorisation of Congo red by *Ceriporia lacerate* ZJSY and decolorization rate was above 90% at 48 h with 3 g mycelia into 20 mL of Congo red solution with the concentration 0.1 mg mL^{-1}.

Fig. 1a. Termitomyces fruiting body

Fig. 1b. Termitomyces isolate

Table 2. Lignolytic enzyme activity of *Termitomyces* sp. (TMS 7)

Days after inoculation	Cellulase activity (µ mol of glucose released/min/mg protein)		Xylanase activity (µ mol of xylose released/min/mg protein)	
	TMS 7	Std	TMS 7	Std
3	5.81	5.21	6.88	6.45
5	6.18	6.18	7.28	7.15
7	6.9	7.2	7.37	7.53
9	6.68	6.98	7.91	8.61
11	9.97	9.8	9.55	9.15
15	8.04	7.3	7.61	7.58
Mean	7.263	7.112	7.767	7.812
Mean SE	0.624	0.626	0.383	0.450
SD	1.529	1.534	0.939	1.103

Std -(Termitomyces albuminosus (MTCC 1366)
SE –Standard error, SD- Standard error deviation

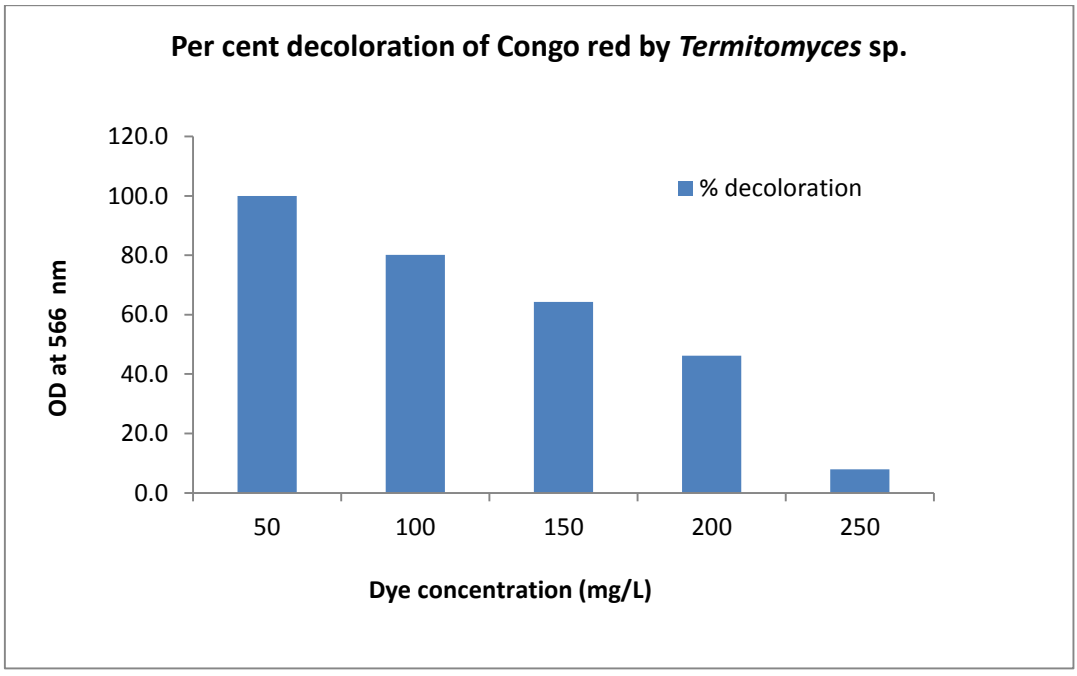


Fig. 2. Effect of *Termitomyces* sp. on congo red decoloration

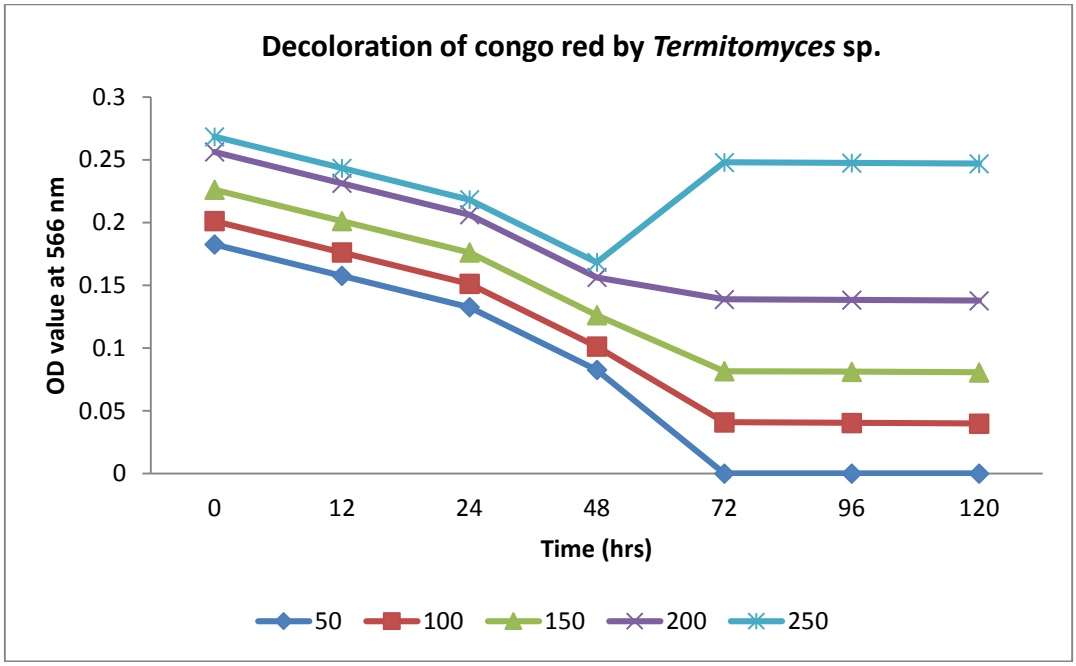
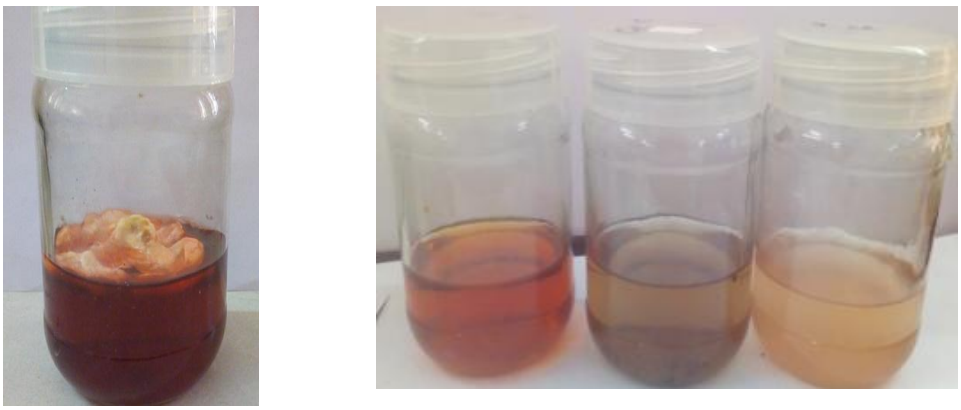


Fig. 3. Decoloration kinetics of congo red by *Termitomyces* sp.



Congo red with fungal mat | **Control** | **Treated with fungal mat (50 mg/L)** | **Treated with fungal mat (100 mg/L)**

Fig. 4. Decoloration of congo red by *Termitomyces* sp. at 72 hrs

The decolorisation kinetics was carried out for congo red at different concentration (50, 100, 150, 200 & 250 mg/L) with Termitomyces fungus. The maximum decolorisation (100%) occurred at a concentration of 50 mg/L after 3 days of incubation. Minimum decolorisation observed was 8 per cent at 250 mg/L dye concentration. From the graph it is inferred that the percent decolorisation increased with increase in the incubation period and the magnitude of the increase was somewhat less in the final stages than that of the initial stages (Fig. 3). This may be due to decline in the rate of enzyme secretion.

Termitomyces sp. fungal mat was found as efficient bio sorption material for decolorisation of synthetic dye congo red (Fig. 4). It was observed that the maximum decolorisation was achieved within 72 h for all the dyes concentrations. Further increase in the incubation period has no significant effect on the decolorisation efficiency.

After 72 hr of incubation, decolored solution again return to colored state, which might be due to the desorption of adsorbed dye again into the aqueous solution. This desorption was high in

high dye concentration (250 mg/L), hence the OD values recorded higher. Similarly OD value of dye concentrations 150 &200 mg/L after 72 hrs were remaining same. The result indicates the fungal inoculum were not able to withhold the adsorbed dye. Therefore after adsorption of dye or other pollutants, biosorbed material will be removed from the solution in a lab scale optimization.

To rectify this issue, optimization study for size of the inoculum required, pH and temperature for decolorisation of synthetic dye is essential. In future optimizing the factors affecting decolorisation efficiency of fungal biomass will be studied in detail to improve the degradation potential.

4. CONCLUSION

According to Heinfling et al. [41] complete mineralization of azo dyes by white rot fungi cannot be expected. It is generally agreed that practical application of biodegradation systems using white rot fungi must be preceded by a better understanding of the biodegradation mechanisms involved. In the present investigation an attempt was made to degrade and decolorize the mostly used textile dye Congo red by white rot fungus *Termitomyces* sp. complete and partial decolorisation was observed in 50 to 250 mg/100 ml dye concentration.

Termitomyces sp. produces ligninolytic enzymes i.e. cellobiose dehydrogenase, xylanase and other lignocellulosic enzymes exudated by the fungus [29,35,36]. Reason behind dye decoloration might be due to the enzymatic degradation and Congo red degraded products might be adsorbed with the cell wall material of fungus. Similar results were earlier reported in which adsorption of CR on the *Aspergillus niger* fungal biomass induced an increase in some peaks intensity and appearance of new peaks due to introduction of new functionalities on the surface of bio sorbent which confirmed the CR adsorption on fungal biomass [18].

This investigation gives insight about the azo dye degradation ability of white rot fungus by fungal sorption method however the nature of degraded intermediates of azo dyes and their biodegradability are not clear yet and need further investigation.

The results indicate that bio sorption and biodegradation of azo dye by *Termitomyces* sp. could be a potential application in the bioremediation of textile effluents containing azo dyes and other toxic compounds with some parameter (pH, temperature, quantity of biomass, organic adsorbents etc.) optimization according to the dye concentration.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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