Preliminary Studies on Laccase Producing Fungi Isolated from Sawmill Waste Dumping Area

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Abstract:

Laccase(E.C.1.10.3.2; parabenzenediol: oxygen:oxidoreductase) belongs to a group of Cu containing polyphenol oxidases, known as multicopper oxidases. In this present study 20 fungal strains were isolated from soil collected from different areas .Out of these only 3 fungal strain showed reddish brown zone around the colony when grown in PDA plates incorporated with 0.02% guiacol. They were tentatively coded as LF-1, LF-2 and LF-3.The most potent fungal strain for laccase production was screened on 0.02% guaicol containing PDA medium. The microscopic morphology of the 3 fungus were studied and also screened the type of medium favorable for better production of laccase enzyme. The culture medium used for the study was Semisynthetic media and Agrowaste Based media. Yeast extract Peptone broth and Glucose peptone broth were used as semisynthetic media. .Of these laccase enzyme productions was found high in yeast extract peptone broth medium. In agro waste based medium of the 3 types of agro wastes used in saw dust incorporated medium production of laccase was found at the 8^{th} day of incubation. The pH of the medium was adjusted to 5.0 before sterilization. Dye decolourisation studies were also conducted to check how far the selected fungus was able to degrade dyes. Of the 4 dyes selected for the study 72% of degradation was found in Methylene blue containing medium .From these preliminary data came to a conclusion that LF-2 might be a good candidate for the production of laccase enzyme.

Keywords: Laccase, guaiacol, congored. methylene blue

Introduction

Laccases are blue multicopper enzymes (EC 1.10.3.2) which oxidize a broad range of both phenolic and non-phenolic substrates, via a four-electron reduction of molecular oxygen to water (1, 2). Laccases are widely distributed in nature and have been isolated from bacteria, fungi and plants and also from lichens and sponges. Literature reviews showed that laccases are widely distributed among the prokaryotes and eukaryotes. For example, laccase from *Azospirillium lipoferem*, *Marinomonas mediterranea*, *Streptomyces griseus*, *E.coli*, *Bacillus subtilis* and many more bacteria have been purified and characterized. Laccase activity has been demonstrated in many fungal species belonging to ascomycetes and basidiomycetes and the enzymes has already been purified from many species. There are many records of laccase production by ascomycetes. Phytopathogenic ascomycetes like *Melanocarpus sp, Cerena unicolor, Mangaportha grisea, Trametes versicolor, Trichoderma reesei* and *Xylaria polymorpha* are examples for laccase production and the enzyme was purified. Besides in plant pathogenic species, laccase production was also reported for some soil ascomycetes, species from the genera *Aspergillus, Curvularia and Pencillium* as well as from some fresh water ascomycetes.

Among physiological groups of fungi laccases are typical of the wood rotting basidiomycetes which cause white rot and a related group of litter decomposing saprophytic fungi, which is the species causing lignin degradation. Almost all species of white rot fungi, were reported to produce laccase in varying degrees and the enzyme has been purified from many species (3).The majority of laccases characterized so far have been derived from white rot fungi which are efficient lignin degraders (4). Many fungi contain, several laccase encoding genes, but their biolological roles are mostly not well understood (5). *Agaricus bisporus* , *Botrytis cineria, Coprinus cinereus, Phlebia radiate, Plaeurotus ostreatus* and *Trametes versicolor* were some examples of basidiomycetes that produce laccase. Interest in laccase has increased recently because of their potential use in detoxification of pollutants and in

bioremediation of phenolic compounds (6, 7 and 8). Laccase exhibit broad range of substrate specificity and have the ability to degrade a range of xenobiotic including industrial colored waste water (9). It is also used in the medical diagnostics and for cleaning herbicides, pesticides and some explosives in soil (10). Keeping in view of the above justification, the aim of the present work was to isolate and screen laccase producing fungi from saw mill waste dumping soil.

Materials and Methods

Materials

All the chemicals used in this study were procured from HI Media, Mumbai, India.

Isolation of fungi

From the collected samples 1gm was weighed and added to 9ml of sterile distilled water and mixed well. The suspension was serially diluted from 10^{-1} to 10^{-7} dilution factors. Later 1ml of each dilution was spread plated on the surface of potato dextrose agar medium plates which contains 0.01%chloramphenicol and incubated at 37^{o}C for 5-7 days. Distinct fungal colonies were isolated and repeatedly sub cultured until pure cultures were obtained. The cultures were maintained on PDA slants at 40^{0}C.

Qualitative screening

Laccase enzyme production was carried out by inoculation of mycelium from each strain into PDA plates containing 0.02% Guaiacol. It was incubated at 37^{0}C for 5- 7 days. The formation of reddish brown zone indicates the positive laccase secretion.

Screening of culture media

Two semi synthetic culture media and 4 types of agro waste based culture media were used for the study.1.Yeast extract peptone dextrose copper sulphate (YPD-Cu)medium contains glucose

20g/l, peptone 5g/l, yeast extract 2g/l, copper sulphate 100mg/l. 2. Glucose peptone broth (GPB) media contain glucose 100g/L, peptone 3g/l, KH_2PO_4 0.4g/l, $FeSO_4$ 0.0005g/l, $MnSO_4$ 0.05g/L, $MgSO_4$ 0.5g/l and copper sulphate 0.01g/l 3.Agrowaste based medium contain 10g of each material(pineapple leaves, wheat bran, saw dust) is individually moistened (70%) with the liquid media containing $(NH4)_2SO_4$ 1g/l and $MgSO_4.7H_2O$ 0.5g/l without glucose (11).

Inoculum preparation

The inoculums was prepared by cutting the 5 days old fungus grown on PDA plates into small discs (10mm) in size and inoculated into 250ml Erlenmeyer flask containing proposed media.

Enzyme activity

The laccase activity was assayed at room temperature by using 10mM sodium acetate buffer (pH 5.0).The reaction mixture contained 3ml acetate buffer,1ml guaiacol and 1ml enzyme source. The change in the absorbance of reaction mixture containing guaiacol was monitored at 470nm for 10 min of incubation using UV – spectrophotometer. Enzyme activity is measured in U/ml which is defined as the amount of enzyme catalyzing the production of one molecule of coloured product per minute per ml (12).

Morphological and microscopical identification

Morphological characterization of fungus was commonly performed to distinguish the microbes based on colony and cellular morphologies. Morphological analysis was performed for colonies of strains on solid media. Size, shape, and staining by lactophenol cotton blue was analyzed using light microscope.

Dye decolourisation

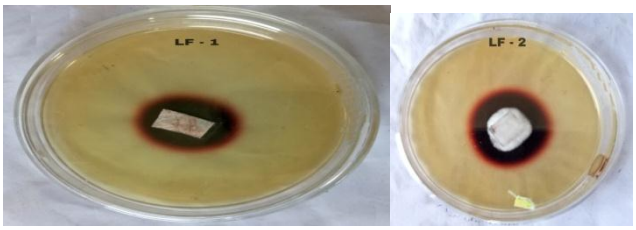
Three synthetic dyes such as Congo red (CR), methylene blue (MB) and crystal violet (CV) were used for investigating the efficiency of deolourisation by the potent fungus .Stock solution of these dyes were prepared in distilled water and diluted to the required concentration and then

used for the decolourisation assay. The reaction mixture contain PDB incorporated with 50mg/l of CR, MB and CV in a total volume of 50 ml. pH was adjusted to 5.5.The inoculum was added and incubated at room temperature. The decolourisations of the tested dyes were calculated at different time interval .The decolourisation rate was calculated using the following equation (13).

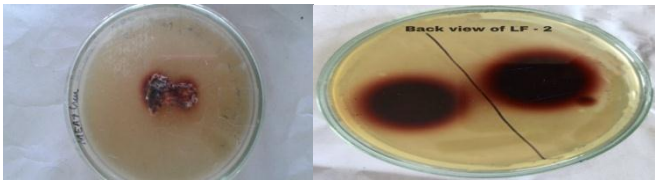
$$\text{Decolourisation Percentage (\%)} = \frac{\text{Final absorbance-initial absorbance}}{\text{Initial absorbance}} \times 100$$

Result and Discussion

A total of 50 fungal colonies were isolated from the saw mill waste dumping soil. Isolated fungi were maintained in PDA medium. They were screened for potential laccase production ability using Guaiacol as indicator. Screening of a large number of fungi is therefore necessary to select strains capable to produce high titers of laccase with normal characters. Such a screening should preferably rely on the use of inexpensive, rapid and sensitive testing methods.(14).The organism surrounded by red brown halo were marked as laccase producing fungi. They were coded as LF-1, LF-2 and LF-3.The diameter of the zones measured was 35mm, 41mm and 12mm respectively.



LF-1 LF-2



LF-3 BACKSIDE VIEW OF LF-2

Figure.1. Reddish brown zone formation around the selected fungal strains LF-1, LF-2 and LF-3.

Of the 2 types of semi synthetic medium used, laccase production was found high in YPD medium. About 2.99 U/ml of enzyme was produced by LF-2 at the 12th day of incubation.LF-1 and LF-2 didn't show a notable drastic change in enzyme production compared to LF-1 (Fig.2 and fig3.).Likewise in the case of GPB enzyme activity was present but the rate of enzyme activity was very low compared to YPD-Cu medium. But in both the medium better activity was noticed by LF2.The presence of copper is an inducing agent for laccase production in the YPD-Cu medium. Copper has been reported as a strong inducer in several species; among them are *Trametes versicolor, Pencillium chrysogenum, Pleurotus* etc. It is known that Cu induced both laccase transcription and activity (15) and increase in activity is proportional to the amount of copper added.

In the case of agro waste based media maximum laccase production was found in medium having saw dust as substrate compared to wheat bran and pineapple leaves. The agro waste act as a nutrient support for the growth of microbes. They are rich in sugars which make the process more economical (16) and help to solve environmental problems which are caused by them due to the disposal in the open environment. The fungus coded as LF-2 showed increase in production of laccase enzyme in saw dust incorporated medium. The maximum activity of about 4.0U/ml (Fig.4) was observed on the 8th day of incubation. In wheat bran

1.08U/ml (Fig.5) of enzyme activity was observed on the 14th day of incubation. In pine apple leaves added medium on 8th day about 3.1U/ml (Fig.6) was observed. So from the above results it is clear that in sawdust incorporated agro waste based medium, better production started at the earlier stages of incubation and the organism was coded as LF-2. So it is selected as a good candidate for further studies.

In the present study LF-2 was identified as *Coriolopsis sp*. The identification of moulds is based on the shape, method of production, and arrangement of spores (conidial ontogeny). White rot fungi belong to the phylum Basidiomycetes and the subphylum Hymenomycetes. Detailed molecular characterization has to do in the next stage of this work.

The dye decolourisation efficiency was checked by inoculating the fungus LF-2 into various dyes like Methylene blue, Congo red and Crystal violet (MB, CR and CV) incorporated medium. On 14th day of incubation observed 72% of degradation in MB incorporated medium (fig.7). No change was observed in the rest other two dyes added medium. Laccase producing microorganisms especially white rot fungi were extensively applied for dyes decolorization experiments. Decolorization ability of five indigenous white rot fungi on vat dyes during 10 days was studied by Asgher et al. (17) and it was determined that *Coriolus versicolor* IBL-04 showed excellent decolorization potential on all tested dyes. Decolorization potential of laccases even on a same dye shows variation and depends on the biological sources of producing microorganism.

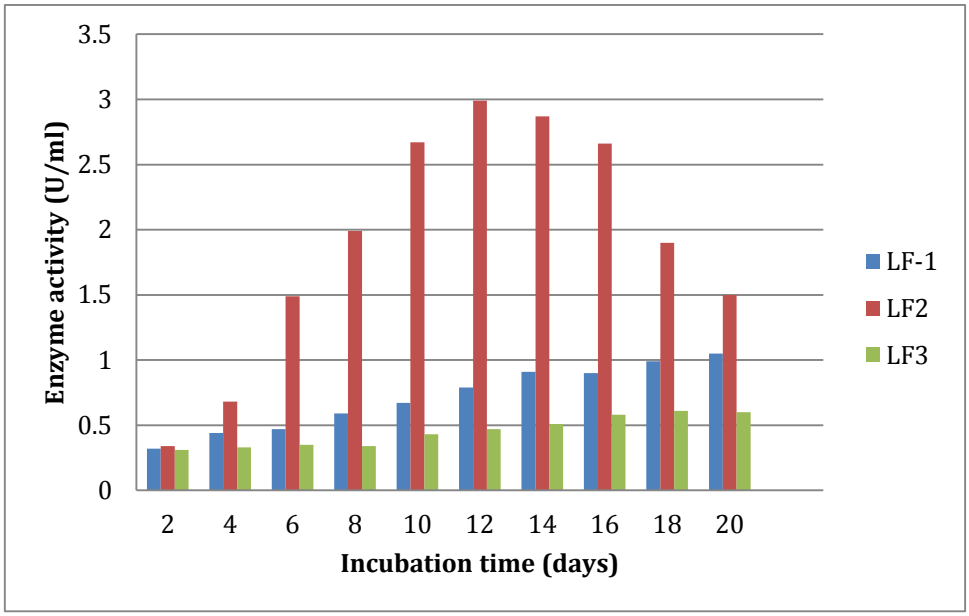


Figure 2. Enzyme activity profile of LF-1, LF-2 and LF-3 on YPD-Cu Broth

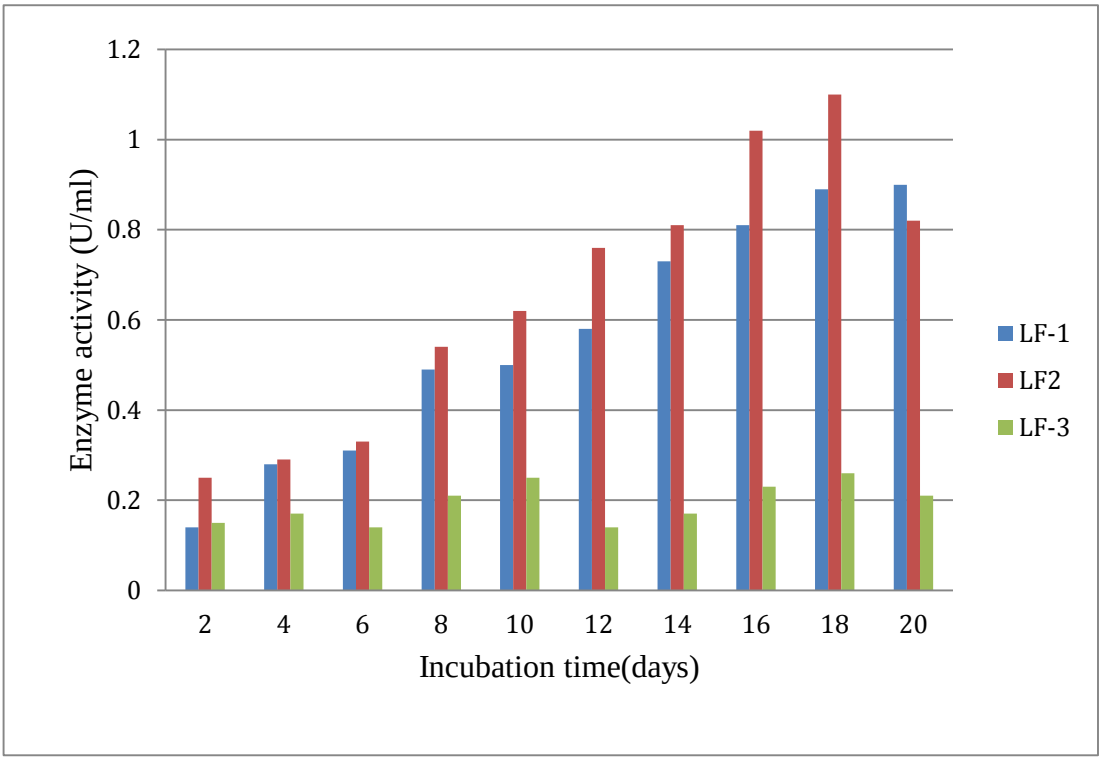


Figure 3. Enzyme activity profile of LF-1,LF-2 and LF-3 on Glucose Peptone Broth

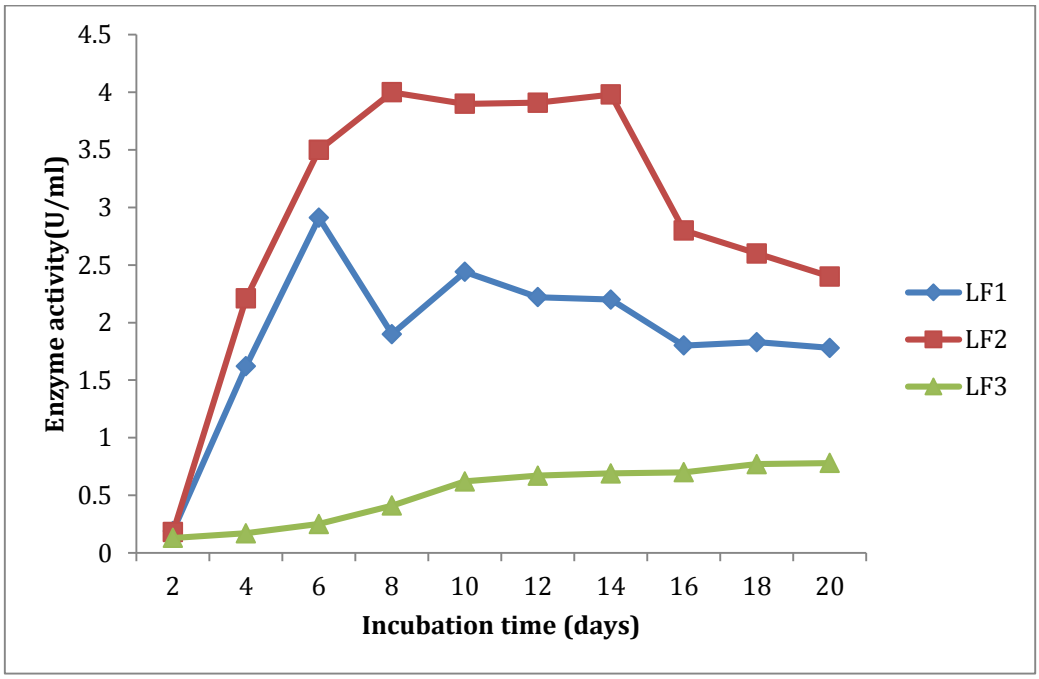


Figure 4. Laccase production by the 3 isolated fungi in Sawdust incorporated medium.

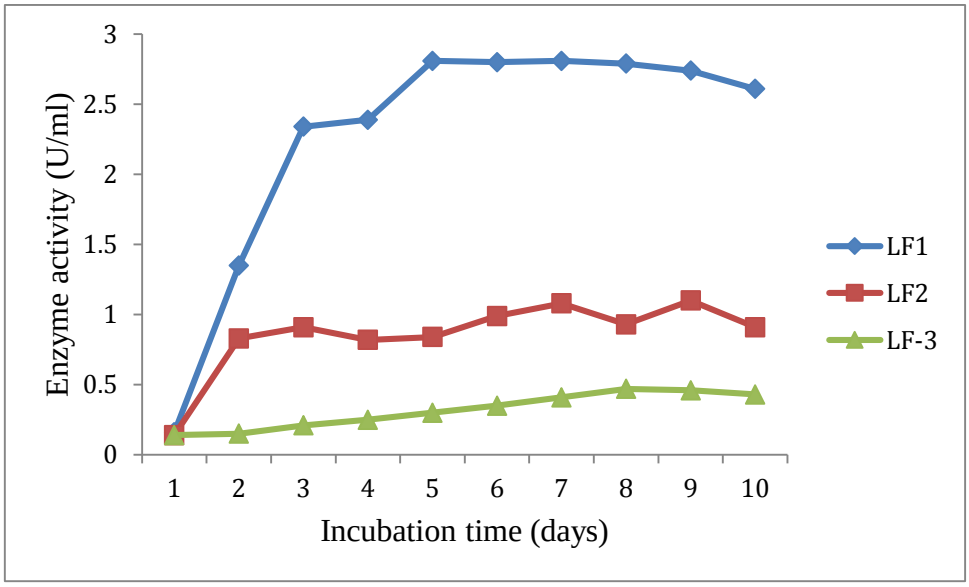


Figure 5.Laccase production by the 3 isolated fungi in wheat bran incorporated medium.

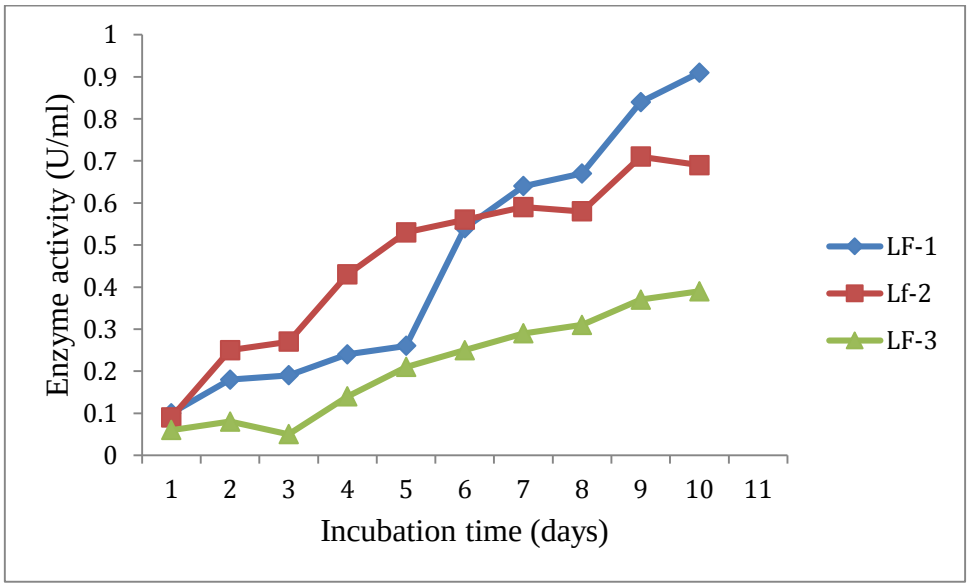
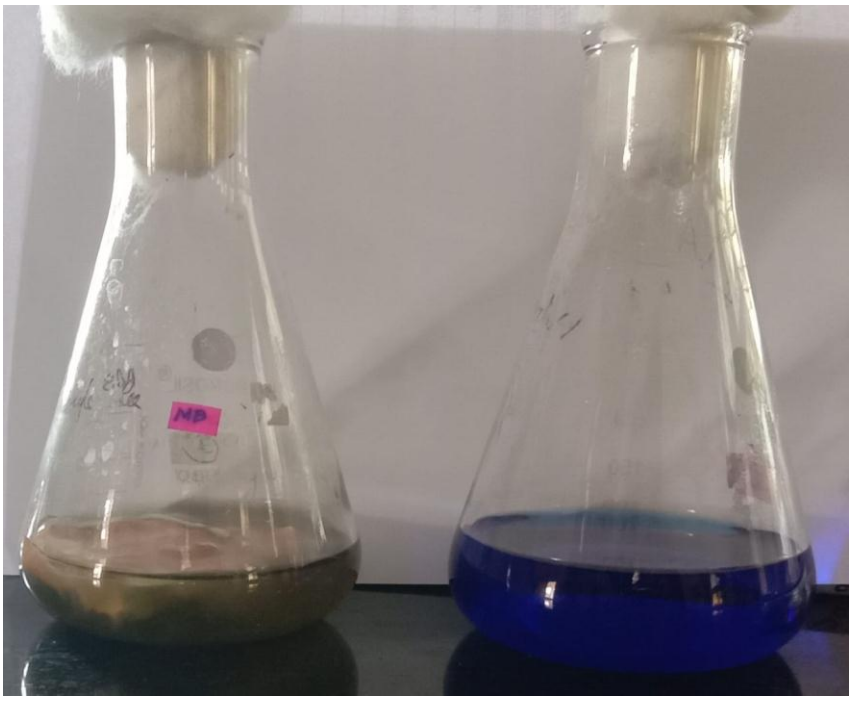


Figure 6.Laccase production by the 3 isolated fungi in pineapple leaves incorporated medium.



colour change observed on 14th day of incubation

CONTROL

Figure 7. Biodecolourisation of methylene blue by LF-2.

Conclusion

From the above studies it is very clear that among the 3 fungal isolates *Coriolopsis sp.* (LF-2 coded fungus) have higher enzyme activity. Guaiacol is found to be a good substrate to facilitate growth and isolation of interested fungi. It can be used for lignocellulosic waste conversion to valuable energy resource. More studies have to be conducted on characterization and purification of this fungal laccase enzyme to explore its application in textile effluent decolourisation.

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References

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