Octa Journal of Biosciences
International peer-reviewed journal
Octa J. Biosci. Vol. 4(2):71-74

ISSN 2321 – 3663
July-Dec 2016

Octa Journal of Biosciences

journal homepage: www.sciencebeingjournal.com

A novel and simple laccase based paper biosensor for the detection of selected pollutants in textile dyeing effluents, water and waste water

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ARTICLE INFO

Received	07 Sept	2016
Revised	15 Nov	2016
Accepted	28 Nov	2016
Available online	19 Dec	2016

Keywords: Biosensors; laccase,;MBTH; phenols

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ABSTRACT

Laccases are multi-copper phenol oxidases, which reduce oxygen to water and simultaneously catalyze the oxidation of aromatic pollutants such as anilines and phenols in water and waste water samples. Based on this catalytic property the enzyme is suitable for the development of laccase based paper biosensors. Conventional procedures for the detection of phenolic compounds are lengthy and expensive. The development of a simple laccase based paper biosensor was evaluated with absorption of laccase from *Trametes versicolor* (UC-3) and mixing with 3-Methyl-2-benzothiazolinone hydrazone hydrochloride hydrate (MBTH) solutions on Whatman No.1 paper. The developed pink shades on biosensors indicated the oxidation products of phenol. The optimum enzyme concentration was 2 mg/mL and MBTH was 24 mM for the detection of phenolic content. The lowest detection limit for phenolic content is 0.1µM. Phenolic content was detected in waters, waste waters, textile dyeing effluents and textile dyes through the laccase based paper biosensor. No report was available for the detection of phenolic content in textile dyes and textile dyeing effluents by using laccase based paper biosensor.

INTRODUCTION

Phenols are present in the environment and are the breakdown products of different plants and animals. Phenols were also discharged from various industries such as resins, plastics, adhesives, iron, steel, aluminum, leather, textile, pulp and paper. Phenolic compounds, a class of organic materials, have a similar structure as of common pesticides, which are resistant to biological degradations (Girelli et al., 2006; Pan and Kurumada 2008).

The effluents containing phenol and its derivatives must be treated prior to their release into the water resources. Furthermore, phenol is a combustible compound that is extremely soluble in water, oil and numerous organic solvents (Ahmaruzzaman, 2008). In addition, in the presence of chlorine in water, phenol forms chlorophenol, which has a medical flavor, which is unpleasant and completely manifested. Also, phenol is a troublesome contaminant that contributes to off the flavors in the food processing waters. Presence of phenol in the water resources results in the reduction of water quality, decrease of aquatic organisms and also prohibition of the common acts of biological community (Lin et al., 2009). Almost the entire phenols are toxic and some are known as carcinogen for human. These compounds are incorporated in the food chain and cause significant environmental troubles. Owing to their toxic effects, including permeabilization of the cellular and cytoplasmic coagulation and also irritation to skin, phenolic compounds can injure sensitive cells and consequently cause profound health and environmental issues (Rengaraj et al., 2002; Gomez et al., 2006). On the other hand, acute poisoning can cause strict gastrointestinal disturbances, kidney malfunction, and circulatory system failure and also lung edema. Deadly doses can be absorbed through the skin. Key organs injured by chronic exposure to phenol consist of spleen, pancreas and kidneys.

Due to the toxicity and persistency of phenolic compounds, their fast monitoring is an important issue. The conventional methods for the determination of phenols include chromatographic and spectrophotometric methods (Townshend, 1995) that are time consuming and complicated processes besides having a low sensitivity. The application of biosensors is promising due to their relatively low costs, specificity, fast analysis and convenience for in field/on site monitoring and minimal sample pretreatment. Laccases and tyrosinases, the two groups of phenol oxidases, have been used extensively in construction of biosensors up to date (Durán et al., 2002). Laccases, a class of copper-dependent phenol oxidases, are produced by plants and fungi (Bollag, 1992; Thurston, 1994). Laccases oxidize aromatic pollutants, such as anilines and phenols, in the presence of oxygen (Bollag, 1992; Bollag et al., 1988; Hoff et al., 1985). In this reaction, the substrates are oxidized by one electron to generate the corresponding phenoxy radicals, which either polymerize to yield a phenolic polymer or are further oxidized by laccase to produce a quinone (Bollag, 1992). Electrons received from the substrate are subsequently transferred to oxygen, which is reduced to water. The use of laccase in biosensor technology is mainly attributed to its broad substrate range allowing for the detection of a broad range of phenolics. Biosensors that utilize laccase include an electrode that may be used for the detection of phenols, such as catechols in tea (Palmore and Kim, 1999), phenolic compounds in wine, and lignins and phenols in wastewaters (Giovanelli and Ravasini, 1993). Fogel and Limson, (2013) developed a rapid, simple method of electrochemically predicting a given phenolic substrate's ability by amperometric laccase biosensors. Several authors detected phenolic compounds in wine, food, tea, lignins and wastewaters using electrode laccase biosensor (Giovanelli and Ravasini, 1993; Palmore and Kim, 1999; Ahmad et al., 2011; Chawla et al., 2011; Shimomura et al., 2011; Fogel and Limson, 2013).

Novel biosensors have been developed using beneficial properties of laccase, such as the potentiometric immuno sensor for the detection of antigens (Giovanelli and Ravasini, 1993). No report was available for the detection of phenolic content in textile dyes and textile dyeing effluent using paper based laccase biosensor. The main aim of this study was to detect phenolic content present in textile dyes and textile dyeing effluent using the developed laccase based paper biosensor. In the present study, MBTH was used for the detection of o-quinones. In the presence of laccase, phenols are oxidized to o-quinone products, which react with MBTH. The pink colour formation allows the detection of phenolic compounds in water, wastewater, textile dyes and textile dyeing effluents.

MATERIALS AND METHODS

Purification of laccase from Trametes versicolor

Laccase was produced from Trametes versicolor under optimum culture conditions. The extracellular culture filtrate was centrifuged at 10,000 rpm for 15 min at 4 °C and collected the supernatant. The collected supernatant was precipitated with ammonium sulphate fractions (0 - 80% w/v). The precipitated protein was then collected by centrifugation at 10,000 rpm for 30 min at 4 °C. The obtained pellet was suspended in a minimum volume of sodium acetate buffer (10 mM, pH 5.5) and dialyzed extensively against sodium acetate buffer (10 mM, pH 5.5). The dialyzed protein was concentrated by lyophilization and used for purification.

DEAE-cellulose column chromatography

The dialyzed sample was loaded onto a DEAE-Cellulose ion-exchange column (10 × 1.6 cm), and washed with sodium acetate buffer (10 mM; pH 5.5). The enzyme was eluted with a linear gradient of 0-0.5 M NaCl in the same buffer. Fractions of 3 mL were collected at every 10 minutes. Active fractions of laccase were pooled separately, desalted, filter sterilized and stored at −20 °C.

Sephadex G-100 column chromatography

The concentrated enzyme from DEAE-cellulose column was loaded onto the Sephadex G-100 column and eluted with sodium acetate buffer (10 mM; pH 5.5) at the flow rate of 3 mL per 10 min. Fractions of 3 mL were collected for every 10 min. Laccase active fractions were pooled separately, dialyzed and concentrated by lyophilization and stored at −20 °C. This laccase was used for the construction of biosensor. MBTH, Guaiacol (2 methoxy phenol), L-DOPA (L-dopamine), Catechol, 4-methoxy phenol were

pooled separately, dialyzed and concentrated by lyophilization and stored at −20 °C. This laccase was used for the construction of biosensor. MBTH, Guaiacol (2 methoxy phenol), L-DOPA (L-dopamine), Catechol, 4-methoxy phenol were purchased from Sigma Chemical Company. Double distilled water was used in all experiments.

Phosphate buffer saline (PBS)

PBS buffer saline pH 7.0 was prepared with (g/L) Sodium chloride 9.95, Potassium chloride 0.25, Disodium hydrogen phosphate 1.14, Potassium dihydrogen phosphate 0.25. Whatman No 1 Filter Paper was cut into 1 cm x 1 cm pieces which were sterilised in an autoclave at 121°C and 15 lbs pressure for 20 minutes.

Construction of paper biosensor

Laccase from Trametes versicolor (UC-3) was dissolved in 100 mL of PBS buffer, pH 7.0 in volumetric flask (100 mL) and considered this as enzyme solution. The laccase enzyme solution (1 mL) was loaded on the Whatman No.1 filter paper (1 cm x 1 cm) and dried in vacuum desiccator for 12 minutes at 37 ± 2 °C. The MBTH solution 1 mL was loaded on the preloaded enzyme paper and dried again in a vacuum desiccator at 37 ± 2° C for 12 minutes, then 20 µl of substrates such as guaiacol (2 methoxy phenol), L-DOPA (L-dopamine), catechol and 4-methoxy phenol were individually loaded and dried for 3 h in a desiccators, maintained at 37 ± 2° C and the paper pieces were observed for the shades of pink (FF0080 Codex). All the substrates dissolved in PBS.

Determination of optimum laccase concentration

The laccase at different concentrations such as 2.0, 4.0, 8.0, 12 and 24 mg/mL in PBS buffer (pH 7.0) were prepared. The laccase enzyme solution (1 mL) was loaded on a Whatman No.1 filter paper (1 cm x 1 cm) and dried in a vacuum desiccator for 12 minutes at 37 ± 2 °C. The MBTH solution (24 mM) 1 mL was loaded on the preloaded enzyme filter paper and dried again in a vacuum desiccator maintained at 37 ± 2 °C for 12 minutes, then 20 µl of guaiacol at various concentrations such as 32, 64, 128, 256, 512 µM were loaded individually and dried for 3 h in a desiccator maintained at 37 ± 2 ºC and the paper pieces were observed for the shades of pink (FF0080 Codex).

Determination of optimum MBTH concentration

The MBTH was prepared in different concentrations such as 6, 12, 24, 48 and 96 mM in PBS buffer (pH 7.0) and were individually loaded on the filter paper pieces. The laccase enzyme solution (2 mg/mL) 1 mL was loaded on the preloaded Whatman No.1 filter paper (1 cm x 1 cm) and dried in vacuum desiccator for 12 minutes at 37 ± 2 ºC, then 20 µl of guaiacol at various concentrations such as 32, 64, 128, 256, 512 µM were reloaded individually and dried for 3 h in a desiccator maintained at 37 ± 2 ºC and the paper pieces were observed for the shades of pink (FF0080 Codex).

Determination of detection limit and sensitivity

The laccase 2 mg/mL and MBTH 24 mM were considered as optimized chemical concentrations and utilized for the detection of phenol such as guaiacol at different concentrations such as 1, 2, 4, 8, 16, 32, 64, 128, 256 and 512 µM were loaded individually on the preloaded Whatman No.1 filter paper pieces (1 cm x 1 cm) and dried in vacuum desiccator maintained at 37 ± 2 ºC for 3 h, then filter paper pieces were observed for the shades of pink (FF0080 Codex).

Determination of phenol and phenolic compounds in water, wastewater, textile dyes and textile dyeing effluents by laccase based biosensor

The laccase (2 mg/mL) 1 mL was loaded on to the Whatman No.1 filter paper and dried in desiccator maintained at 37 ± 2 ºC for 12 min and then MBTH 24 mM was loaded on to the preloaded enzyme filter paper and again dried in desiccator maintained at 37 ± 2 ºC for 12 min.

Different water samples such as tap water, bore well water, pond water, distilled water collected from MCRC, drinking water (Commercial), drinking water (Home), sewage water (Taramani canal) were collected in different clean glass containers and brought to the laboratory, The textile dyeing effluent E II and III, textile synthetic dyes such as Red ME4B, Black B were collected from Textile dyeing industry Erode, Tamil Nadu, The liquid phenol (SRL, AR 99% purity) and phenol 1µM conc. and the 11 waters and waste waters listed above were individually tested for the detection of phenol and phenolic compounds in a laccase based paper biosensor. The commercially available liquid phenol (99%) and crystal phenol at 1 µM concentration were used as the standard.

RESULTS

Detection of phenol and phenolic compounds using laccase based paper biosensor

The development of a laccase based paper biosensor was evaluated with absorption of laccase from *Trametes versicolor* (UC-3) and mixing with MBTH solutions on Whatman No.1 paper. After the absorption of laccase on Whatman No.1 paper, developed the light yellow shade. After absorption of MBTH, development of yellowish green was observed on preloaded laccase Whatman No.1 paper. The mixture of laccase and MBTH developed a thick yellowish green background on the Whatman No.1 paper. Hence, laccase and MBTH were loaded separately and avoided the formation of background thick yellowish green shade.

Optimum enzyme concentration

The laccase based paper biosensor developed a pink shade over the range of guaiacol and enzyme concentrations tested (Fig.1). Among 4 concentration of laccase, 2 mg / mL of laccase developed low background yellowish green on the preloaded MBTH Whatman No.1 compared to no substrate control. At higher laccase concentrations, background colour interfered with visual detection of the pink shade.

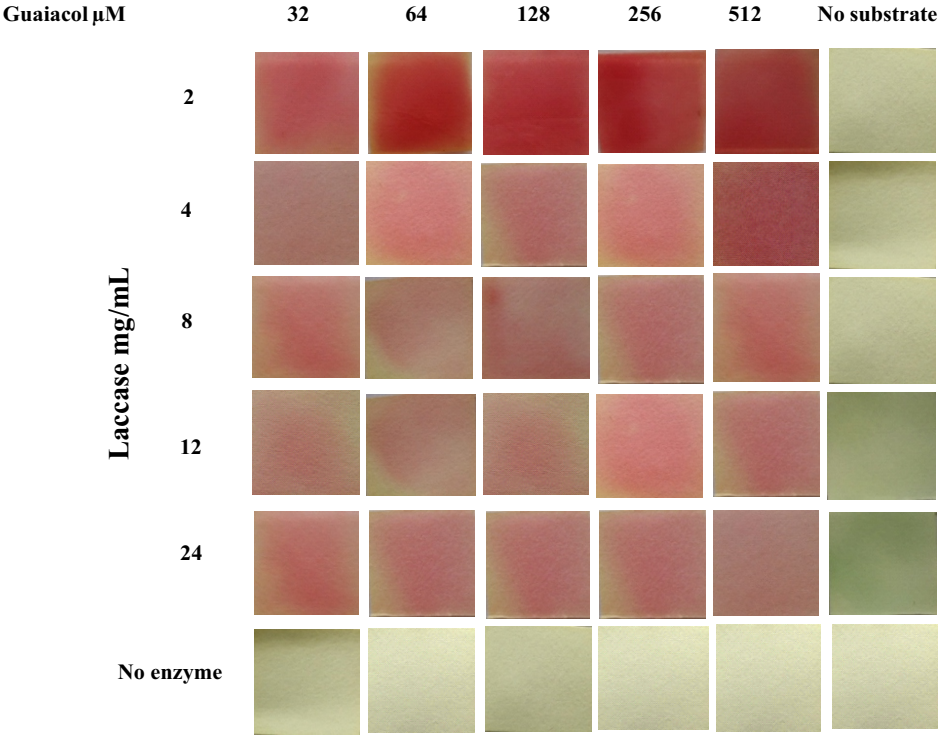


Figure 1. Optimization of enzyme concentration for laccase based paper biosensor where in guaiacol loaded at various concentrations and the MBTH concentration was at 24 mM. The papers at bottom row were not loaded with enzyme (No laccase). Only PBS was loaded to no substrate controls (No substrate) (rightmost).

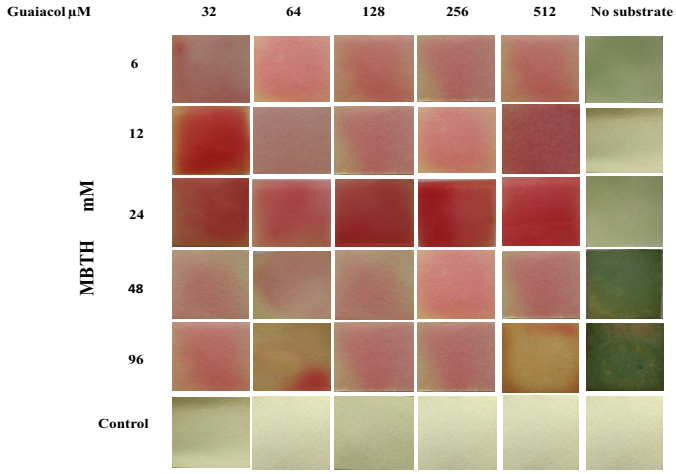


Figure 2. Optimization of MBTH concentration for laccase based paper biosensor where in guaiacol loaded at various concentrations and the laccase was at 2 mg/mL conc. No substrate represents papers loaded only with MBTH- laccase mix and Control represents papers loaded only with guaiacol).

Optimum MBTH concentration

The effect of MBTH concentration on preloaded laccase Whatman No.1 paper was investigated Among 5 concentrations of MBTH, 24 mM MBTH has resulted in the development of the lowest background hence considered as optimum concentration of MBTH (Fig. 3).

Optimum substrate concentration

The Whatman No.1 filter paper containing laccase (2 mg / mL) and MBTH (24 mM) were loaded with different concentration of guaiacol. In all concentrations (0.1 to 512 µM) of guaiacol tested has developed a pink shade on laccase based paper biosensor which indicated the presence of phenolic compounds and at 0.1 µM guaiacol was recorded as the lowest detection limit (Fig. 3).

Phenols and phenolic compounds in water, waste water and textile dyes and dyeing effluent on laccase based paper biosensor

Among 11 samples tap water (MCRC), bore well water (MCRC), pond water (MCRC), sewage water (Taramani canal), textile dyeing effluents (E II, E III) and textile synthetic dyes such as Red ME4B, Black B contained phenol detected on laccase based paper biosensor (Table 1). The commercially available liquid phenol (99%) and crystal phenol at 1 µM concentration developed dark pink shades to the light pink shades on the laccase based paper biosensor which indicated the presence of the phenol. The other phenolic compounds such as L-DOPA, catechol and 4-methoxy phenol at 0.1 µM tested have developed the shades of pink on laccase based paper biosensor which indicated the presence of phenolic compounds (Table 1).

DISCUSSION

A number of biological components including microorganisms, enzymes, antibodies, antigens and nucleic acids can be used for the construction of biosensors for the detection of the phenolic compounds (Karim and Fakhruddin, 2012). The electrochemical oxidation of the hydroquinone formed in the biodegradation of p-nitrophenol and 20 nM was measured using Moraxella sp. modified carbon paste electrode (Mulchandani et al., 2005). Purified enzymes have been most commonly used in the construction of biosensors due to their analytical specificity (Darsanaki et al., 2013). Several reports highlighted the detection of phenols and lignins in different samples such as tea, wine and wastewaters using laccase based electrode biosensor. Novel biosensors have been developed as the potentiometric immunosensor for the detection of antigens. (Giovanelli and Ravasini, 1993; Edens et al., 1999; Palmore and Kim, 1999; Kuznetsov et al., 2001; Fogel and Limson, 2013).

An amperometric biosensor was developed for the determination of the polyphenolic compounds based on the immobilization of laccase (Gomes and Rebelo, 2003). In the case of laccase-based biosensors, an extensive research effort has been addressed to immobilize laccase on surfaces by different immobilization strategies in order to design biosensors with a wide range of applications (Durán et al., 2002; Gutiérrez-Sánchez et al., 2012). Laccase has been immobilized by the direct adsorption, covalent binding or entrapment onto epoxy resin membranes (Chawla et al., 2011) mesoporous materials with well-controlled pore structures (Xu et al., 2010; Shimomura et al., 2011), nanocomposites formed by chitosan and carbon nanotubes (Diaconu et al., 2011), copper-containing ordered mesoporous carbon chitosan matrix (Xu et al., 2010), pyrenehexanoic acid-modified hierarchical carbon microfibers/carbon nanotubes composite electrodes (Sánchez-Amat et al., 2001), polyvinyl alcohol photopolymers, sol-gel matrix of diglycerylsilane (Montereali et al., 2010), 3-mercaptopropionic acid self-assembled monolayer modified gold electrodes, cysteine self-assembled monolayer and quantum dots modified gold electrodes (Wang et al., 2009), nanocomposites of silver nanoparticles and zinc oxide nanoparticles electrochemically deposited onto gold electrodes (Chawla et al., 2012), platinum nanoparticles and reduced graphene composites deposited onto screen printed electrodes (Eremia et al., 2013), polyethyleneimine coated gold-nanoparticles modified glassy carbon electrodes (Brondani et al., 2013) and multi walled carbon nanotubes (Cesarino et al., 2013).

In the present study the purified laccase from Trametes versicolor (UC-3) was utilized for the development of a simple, cost effective, portable and disposable paper biosensor. After the absorption of laccase on Whatman No.1 paper, developed the light yellow shade. After absorption of MBTH, development of yellowish green was observed on the preloaded laccase Whatman No.1 paper.

Two mg / mL of laccase, 24 mM MBTH developed a low yellowish green background and developed a high pink shade on the biosensor. Similar results were reported by Oktem et al. (2012). The laccase based biosensor was evaluated for the determination of phenol and phenolic compounds in various samples such as water, wastewaters, textile dyeing effluents and dyes. Among 11 water samples tested for the presence of phenol, tap water (MCRC), bore well water (MCRC), pond water (MCRC), sewage water (Taramani canal), textile dyeing effluents (E II, E III), and red ME4B textile dye contained phenolic compounds detected on laccase based paper biosensor. The commercially available liquid phenol (99%) and phenol at 1 µM concentration tested on laccase based paper biosensor indicated and confirmed the presence of phenol by the development of pink colour. The phenolic compounds such as L-DOPA, catechol, guaiacol and 4-methoxy phenol were detected using the laccase based paper biosensor. The lowest detection limit of the laccase based paper biosensor was in the range 0.1 µM of phenol and phenolic compounds. Oktem et al. (2012) developed a laccase and tyrosinase based paper biosensor using a paper strip absorption method and determined 4-chlorophenol, catechol (0.032 mM) and 0.128 mM of m - cresol and p-cresol.

This biosensor is economic, convenient and easy for the non-experts to use when compared to the other biosensors. The biosensor developed in this study is effective in the detection of phenolic pollutants in different waste waters and textile dyeing effluents. No report was available for the detection of phenolic content in textile dyes and textile dyeing effluent using paper based laccase biosensor. Detection of phenolic content using biosensor is commercially feasible.

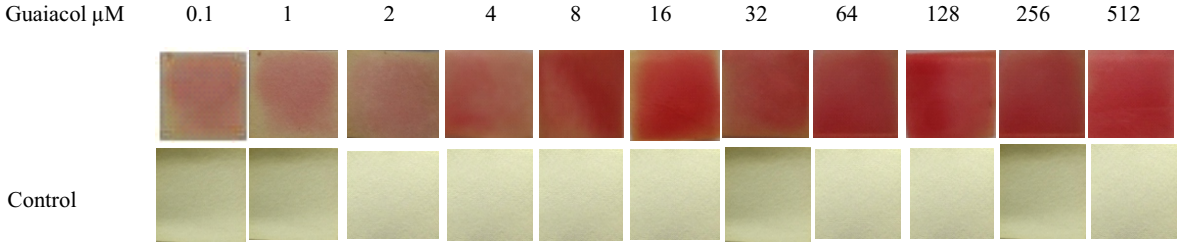
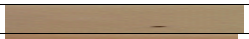


Figure 3. The detection limit of laccase based paper biosensor for guaiacol Phenols and phenolic compounds in water, waste water and textile dyes and dyeing effluent on laccase based paper biosensor.

Table 1. Phenol content in different samples tested through laccase based paper biosensor.

Samples		Shades developed on laccase based paper biosensor	Phenol content (µM / mL) tested through laccase based paper biosensor
Waters / Wastewaters	Metro water (MCRC)		1
	Bore well water (MCRC)		2
	Pond water		512
	Distilled water		0
	Drinking water Commercial source		0
	Drinking water (Home)		0
	Sewage water		4
Effluents	Textile Dyeing Effluent E II		16
	Textile Dyeing effluent E III		4
Textile Dyes	Synthetic textile dye Red ME4B,		8
	Synthetic textile dye Black B		0
Phenols/ Phenolic compounds	Liquid phenol (99%) SRL		›512
	Phenol 1µM		1
	Catechol (1µM)		1
	L-DOPA (1µM)		1

Acknowledgements

The authors thank Shri A.M.M. Murugappa Chettiar Research Centre for providing laboratory facilities to carry the research activities. I would like to thank Department of Science and Technology for their financial support (DST/TSG/NTS/2010/75).

Disclosure statement: No potential conflict of interest was reported by the author.

Financial and proprietary interest: Nil

Financial support: This research was financially supported Department of Science and Technology for their financial support (DST/TSG/NTS/2010/75).

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