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Optimization of Laccase Enzyme Production by *Amesia atrobrunnea* A2: A First Report

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Lignin is one of the recalcitrant compounds largely present in enormous amount on earth. It is considered as major paper industries pollutant because of its brown color and hydrophobicity. Laccase is one of the major lignin degrading enzymes for solution of recent environmental problem. A study was carried out on fungi to establish laccase production potential for industrial and environmental application. The present research for laccase activity was reported by screening 16 isolates from rotted wood samples and agro waste collected from Patan, Gujarat. One of the isolated fungal species showing highest production of laccase enzyme activity was identified to be *Amesia atrobrunnea* A2. The objective of this work was to isolate laccase producing fungal isolate and optimize the production of laccases by *Amesia atrobrunnea* A2 in submerged fermentation. Among six variables (temperature, pH, carbon, nitrogen, inducer and cation sources), glucose as carbon source and veratryl alcohol as inducer were identified as good enhancer of laccase production.

Keywords: Agro waste, Biomass, Guiacol, Lignin, Rotted wood.

Majorly cellulose, hemicelluloses and lignin are three components involved in structure of lignicellulosic material. In nature, cell wall has lignin as a biopolymer, a major and essential component of wood, imparting rigidity and protecting the easily degradable cellulose from attack by pathogens[1, 2]. Because of complication of structure and avaibility of nonhydrolysable bond, leads difficulty in breakdown of lignin compare to cellulose and hemicelluloses. Much of destruction of wood observed in nature is due to lignin biodegradation and it may have an important role in plant pathogenesis. Lignolytic microorganism and their enzymes have become important because they may help to protect environment from pollution by paper pulp and various other industries[3, 4].

Yoshida first described laccase in 1883 when he extracted it from the exudates of the Japanese lacquer tree, *Rhus vernicifera.* Laccase (1.10.3.2, p-diphenol: dioxygenoxidoreductases) belongs to group of enzymes called the blue multi copper polyphenol oxidases (PPO), most widely distributed of all the large blue copper containing proteins. It shows its presence in a wide range of higher plants and fungi[5, 6, 7, 8, 9]. Laccase with wide substrate utilization capacity helps to provide practical application for industrial purposes and/or bioremediation processes of polluted environmental

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area[3]. Laccase has simple requirement for catalysis (presence of substrate and O_2) which shows its great stability and sustainability against inhibition (as has been observed with H_2O_2 for peroxidase) helps to use this enzyme in attractive area of biotechnological applications[7].

In 1896, laccase was demonstrated to be present in fungi for the first time by both Bertrand and Laborde[5]. Since then, Ascomycetes, Deuteromycetes and Basidiomycetes are known for presence of laccase in their cell; being particularly abundant in many white-rot fungi that are involved in lignin metabolism. In biotechnological application, fungal laccase is more important compare to bacterial or plant laccase because of their high redox potential (up to+800 mV). Thus, the degradation of lignin or in the removal of potentially toxic phenols arising during lignin degradation completed with fungal laccase[5, 10]. Generally bacteria are less considered for lignin degradation because they have less lignolytic activity and more cellulolytic activity[11]. Laccase has less substrate specificity and has importance in degradation of xenobiotics including industrial colored wastewaters, dyes and effluents from paper pulp mill which are recognized as ecological pollutants[12, 13].

In present study, ligninolytic microorganisms were isolated from a decayed wood. In order to select microorganisms that could be useful for disrupting the lignocellulosic matrix, qualitative plate assay was applied. One of the isolates was selected which shows brownish halo around colony after primary screening. Optimization of different physical factors was carried out to increase production of laccase enzyme.

MATERIAL AND METHODS

Total 5 rotted wood samples and 25 agrowaste samples were collected from Patan, North Gujarat, India. The Fungal isolates were screened at primary stage with transfer in lignin containing media for 7- 10 days where lignin was main source of carbon with 1% glucose to check the dephenolization ability of isolates. For secondary screening, selected isolates were observed by phenol oxidase production ability. The isolates were grown on potato dextrose agar with 0.02% guiacol as a substrate. The medium was inoculated with fungal culture and incubated at 30°C for 7-10 days[14, 15]. Organisms with brown halos formation were selected and subcultured for further optimization of laccase enzyme.

The quantitative determination of laccase activity, guaiacol oxidizing strains were grown in 250 ml Erlenmeyer flasks containing 100 ml of kirk's medium, composed of (g/l) ; KH_2PO_4 0.20, $CaCl_2$ 0.01, $MgSO_4.7H_2O$ 0.05, ammonium tartrate 0.22, 2.2-dimethylsuccinic acid 2.90, glucose 5, thiamine 0.1, tween 80 0.10% v/v, veratryl alcohol 1.5 mM, and a mixture of trace elements composed of (mg/l): $MnSO_4$ 33, $Fe_2(SO_4)_3$ 50, $ZnSO_4.7H_2O$ 43, $CuSO_4.7H_2O$ 80, H_2MoO_4 50 was used[4, 16]. Each flask was inoculated with six cylindrical plugs (8 mm in diameter) of active mycelia from previously cultured in malt extract agar and incubated at 30°C, 120 rpm for 9 days. Samples were taken at regular intervals and centrifuged at 9500 xg, +4°C for 10 min. The supernatants were used to evaluate enzyme activity[7, 17]. Effects of optimum conditions for temperature, pH, carbon, nitrogen source, inducers and cations on ligninolytic enzyme production were conducted in triplicate and cultured for 9 days. One ml sample of the supernatant from each flask was taken and assayed for laccase activity[18].

Extracellular laccase (EC 1.10.3.2) production was determined by monitoring the A420 change related to the rate of oxidation of 50 mM ABTS (2, 2-Azino- Bis-3- ethyl-benzthiozoline-6-sulphonic acid) in 100 mM Na-Acetate buffer (pH 5.0) at 420 nm and an extinction coefficient of 36000 M^{-1} cm^{-1} was used for analysis[19, 20, 21]. Activity determined in this study was expressed as U/mL against a suitable control. Laccase activity was defined in terms of one unit as the amount of the laccase that oxidized 1 µmol of ABTS substrate per min. Protein concentration was measured by the Bradford assay with dye reagent[21]. Fungal mycelia was separated and washed three times with water. The mycelia were dried at 60°C to constant weight formation[22].

RESULT AND DISCUSSION

Extracellular laccase activity was observed in sixteen fungal isolates. The isolates examined for production of brown color halos

around fungal mycelium growth on guiacol containing PDA plate, which is characteristics of phenol oxidase production on solid medium by isolate (Fig. 1).

The isolate designated as A2 was found to be the best amongst the sixteen isolates. Thus this screening helps us to select a promising fungal isolate. The strain grew well, covering entire plate within seven day incubation in PDA plate. It shows reduced mycelium growth when incubated with guiacol containing solid medium, which may be due to changes of some metabolic activity of isolates during growth in selected media.

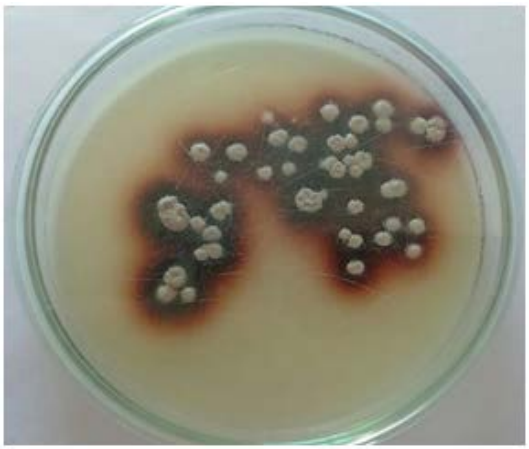

Fig. 1. Guiacol oxidation by *Amesia atrobrunnea* A2

The identification of A2 was further corroborated with studies on its 18S rRNA sequencing carried out by Chromus biotech, India. The isolate was identified as *Amesia atrobrunnea* A2. The phylogenetic tree was structured by neighbour joining (N-J) method using molecular evolutionary genetics analysis (MEGA 6) software (Fig. 2). The sequence was deposited in the GenBank database (Accession no. MF348244). BLAST analysis of the amplificons indicates that, it had the highest similarities (over 99%) sequences generated from *Amesia* strains. To clarify the phylogenetic position of A2, a phylogenetic tree was constructed based on the 5.8S rDNA sequences. The result revealed that A2 strain was closely related to *Chaetomium nigricolor*.

Selection of different parameters helps to optimize laccase production. Temperature is major key factor for laccase activity and growth of microorganisms. Due to respiration, there is general increase of temperature observed. Though its major impact is observed during scale up process at industrial level, it observed as important factor during enzyme production and fungal growth[25]. Normally many fungal isolates have maximum enzyme activity at 30°C but *Amesia atrobrunnea* has maximum laccase activity at temperature of 35°C with good growth (Fig. 3). Yadav *et al*. (2019)

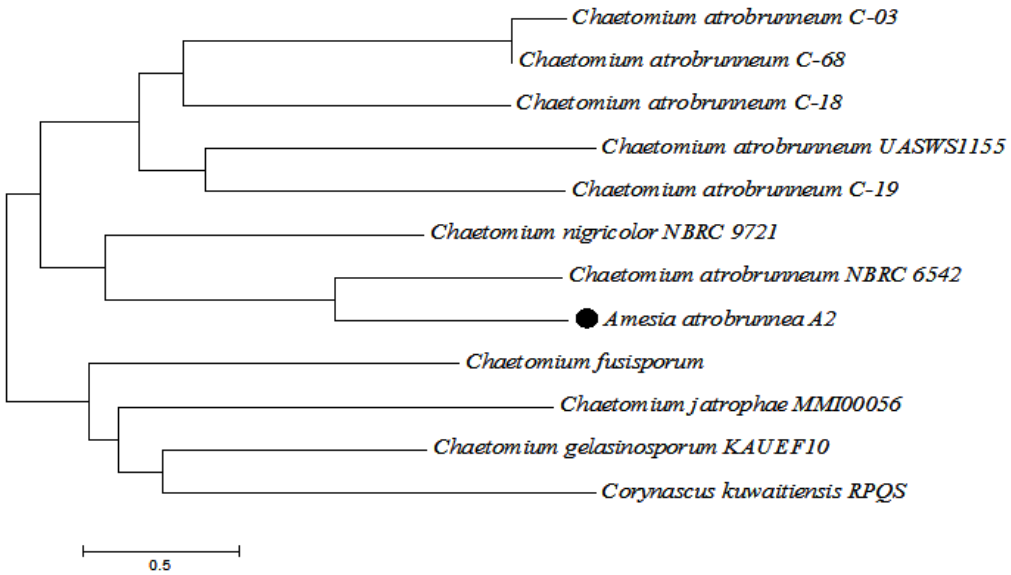

Fig. 2. Phylogenetic relationship of *Amesia atrobrunnea* A2

and Sara *et al.* (2016) have also examined laccase production at 35 °C temperature for *Chaetomium species* [23, 24]. While Abdel-Azeem and Salem, 2012 have observed growth of C*haetomium globosum* at 30 °C[25]. Asgari and Zare (2011) observed range of temperature (30 to 45 °C) for growth of different species of *chaetomium*[26].

Fungal cultivation is majorly affected by pH, one of the crucial parameter for fungal cultivation. The optimum pH for laccase oxidation was 6.5 and further reduction of production was observed at high pH. This organism has low activity with pH lower than 5.5. It may have optimal pH range of 5.5 to 7 (Fig. 4). This may be due to alteration of catalytic site or structure of enzyme with variation in pH[26]. Chefetz & Hadar (1998) obtained similar result for laccase production with optimal pH range about 6 to 8 using *Chaetomium thermophilium*[27]. Many researchers also examined optimum laccase activity at pH 5 to 7 [24, 25].

Conventional carbon and nitrogen sources on adaptation of fungus are important and for

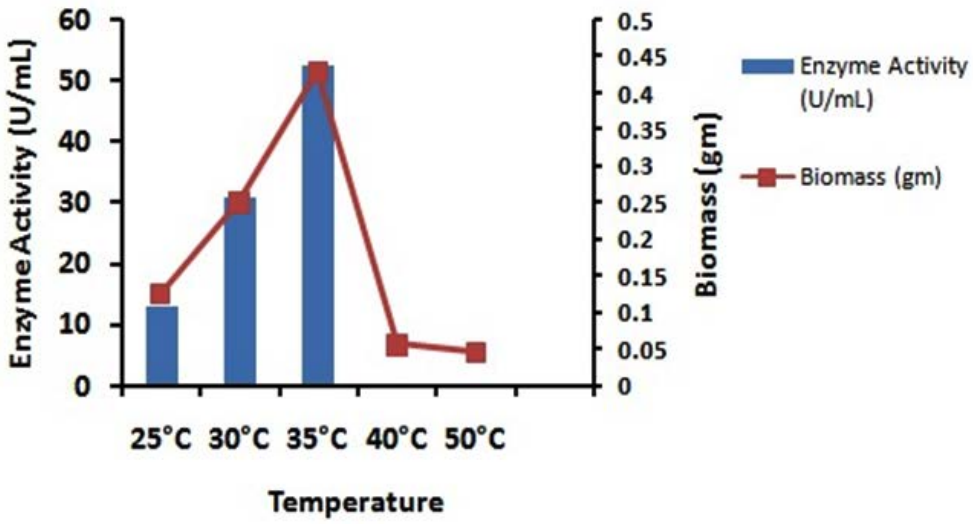

Fig. 3. Effect of temperature on Laccase and Biomass Production

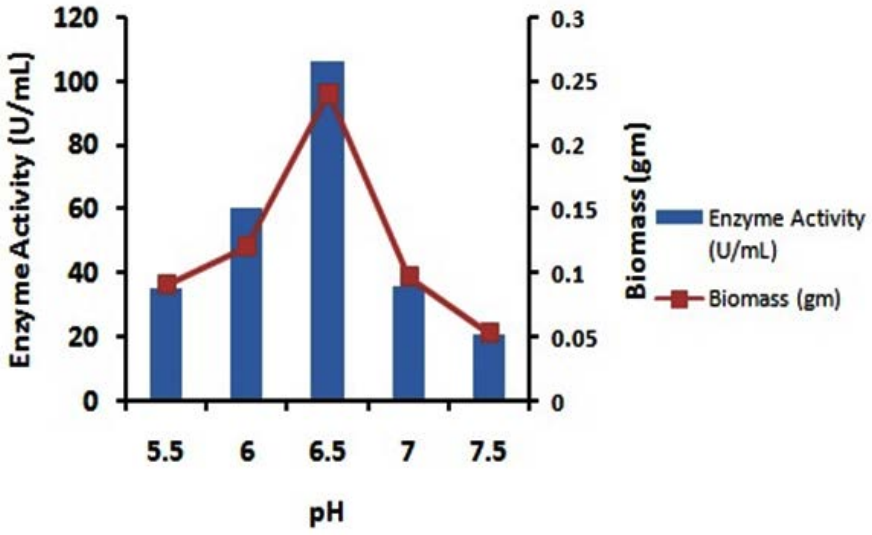

Fig. 4. Effect of pH on Laccase and Biomass Production

production of laccase in suitable amount. Nature and concentration of nitrogen source may increase lignolytic enzyme production and act as powerful nutritional factor for wood rotting fungi[28]. Glucose supplementation helps to increase lignolytic enzyme production and growth of organism to degrade lignin present in solid lignocellulosic substrate[29]. Different sugar sources show different laccase activity. Present study showed higher laccase production with glucose (1 %) by *Amesia atrobrunnea,* which may had effect on growth of organism (Fig. 5). It also showed good laccase activity with use of sucrose (1 %), might indicates alternative use of waste molasses in industrial production laccase production. While maltose also reported as inducer for laccase activity by Abdel-Azeem and Salem, 2012 [25].

Different source of nitrogen also helps to induce laccase activity with use in minimum amount. *Amesia atrobrunnea* with 2.4 mM ammonium nitrate showed significant effect on laccase activity. Ammonium sulphate also had remarkable effect on laccase activity (Fig. 6). D'Souza et al. (2006) observed different variety of nitrogen source have also impact on decolorization of effluents[30]. Umikalsom *et al.* 1997 have examined cellulase production from *Chaetomium* species by using peptone as nutritional factor [31].

The production of laccase was induced by use of aromatic compounds (Such as Veratryl

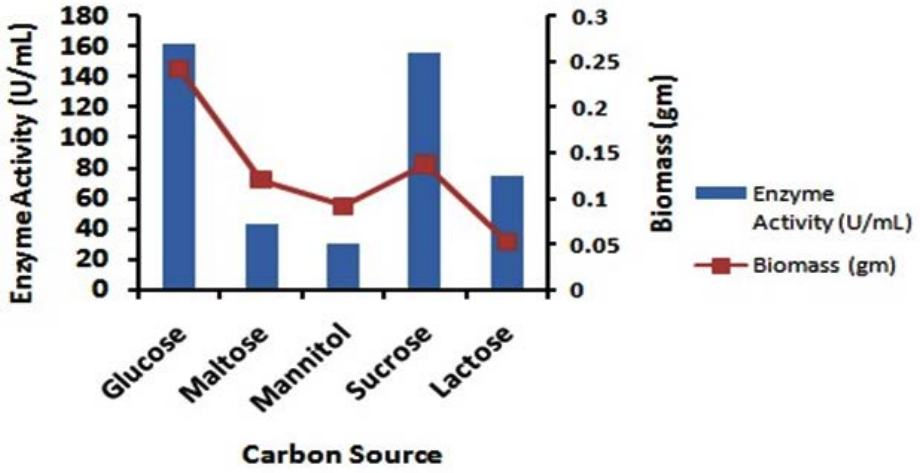

Fig. 5. Effect of various Carbon source on Laccase and Biomass Production

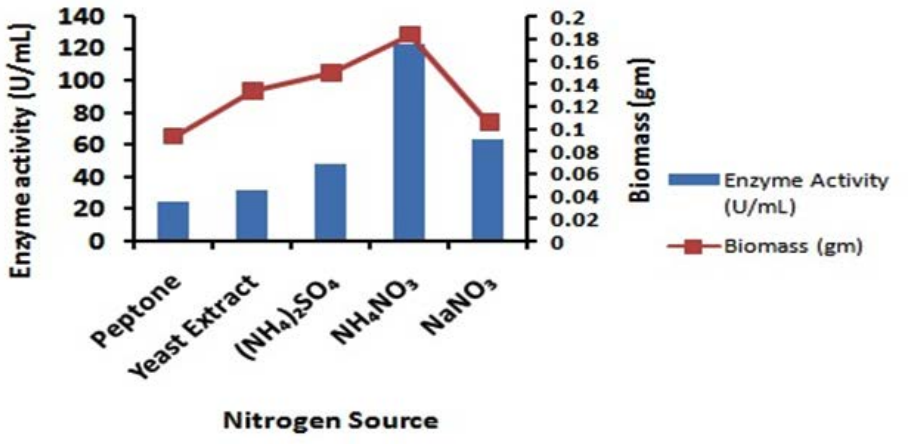

Fig. 6. Effect of various Nitrogen source on Laccase and Biomass Production

alcohol, syringaldazine, Thimine HCL, 2, 6 Dimethoxy phenol and Lignin). The inductive effect of veratryl alcohol at 1.5 mM concentration was observed during optimization process (Fig. 7). 2, 6- Dimethoxy phenol and syringaldazine also showed positive effect on laccase production, but it could not show its role on growth of fungi. Arora and Gill (2001) has also reported influence of veratryl alcohol on laccase production in *Dichomitus squalens, Ganoderma lucidum* and *Trametes versicolor*[32]**.**

The laccase activity was 119, 102.45, 90.66 U/mL respectively with Fe^{+2}, Cu^{+2} and $Zn^{+2}$with 80 ppm concentration after 9 day of incubation (Fig.8).

Laccase expression can be regulated by use of selected metal ions. Different species have different affinity with different metal ions.

Laccase activity in almost all fungi can be influenced by copper in *Trametes versicolor*, *Ceriporiopsis subvermispora*, *Pleurotus ostreatus* and *Trametes pubescens* [33, 34]. The copper-mediated regulation of laccase occurs at the transcription level[22].Laccase activity significantly increased in the presence of Fe^{+2} in *Pleurotus eryngii*[35] and in *Trametes velutina* [36].

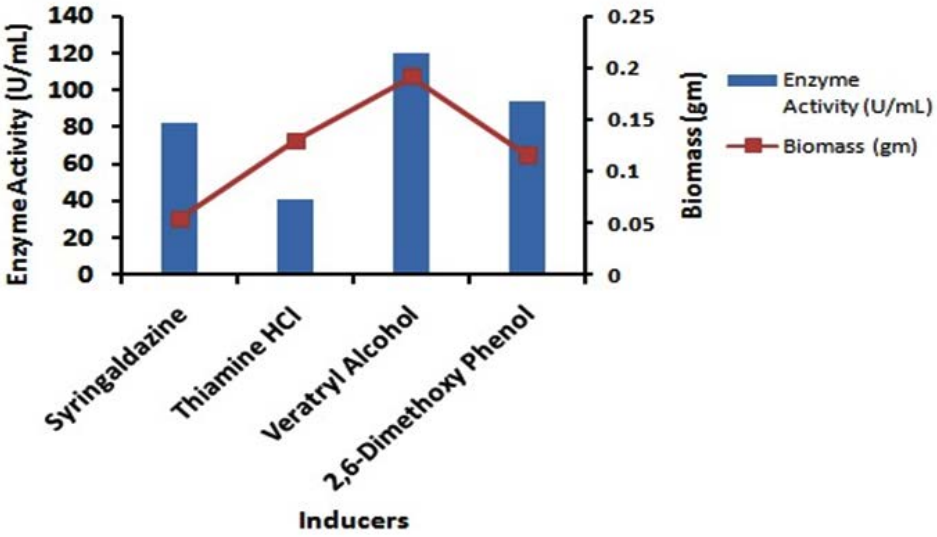

Fig. 7. Effect of Organic Inducers on Laccase and Biomass Production

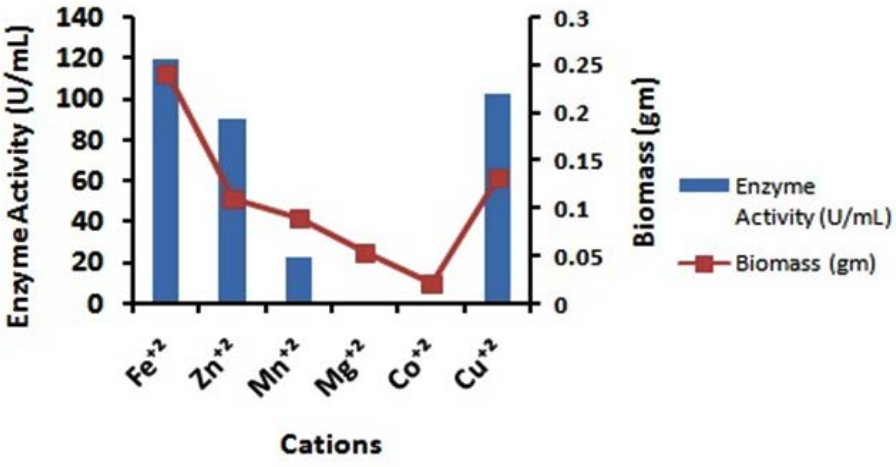

Fig. 8. Effect of Inorganic Cations on Laccase and Biomass Production

CONCLUSION

Lignocellulose is major bio waste available on earth which can become major problem in terms of smell, area occupation and increase in number of vectors. Lignolytic enzymes help to solve this problem. The present study aimed at isolation of laccase producing fungal isolates and screening for their ability to produce laccase with help of lignin. *Amesia atrobrunnea* is a genus of filamentous fungi that are commonly found in soil and on decaying vegetation. The main purpose of the screening was to select fungi with desired characteristics intended for various industrial and environmental applications for example, dye decolorization and lignocellulolytic enzyme production. The reddish brown halos surrounding the mycelia on the plate with guaiacol supplementation was an indication of the presence of laccase activity produced by *Amesia atrobrunnea.*

Optimization of the fermentation media by conventional method helps to increase laccase activity. The enzyme activity could be enhanced by supplementing various cations like Fe^{+2}, Cu^{+2} and veratryl alcohol as inducer with glucose as carbon source. Here, inducer had maximum effect on laccase activity compare to other physical parameters. It has optimum enzyme activity with ammonium nitrate as nitrogen source at 35°C temperature; pH 6.5 of media. Further study is required to check whether any synergistic effect was observed using different combination of media factors. In general the study underlines the need to explore more organisms with different composition of media to express and evaluate the real potential of laccase production by fungi.

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