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Effect of Crude Oil on Biomass Production, Polysaccharide, and Polyphenol Content of *Leucocoprinus cretaceus* (Bull.) Locq. a white rot fungus

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ABSTRACT: There is need to establish the best lignocellulosic wastes with bio-stimulatory effects on mushrooms been investigated for myco-remediation purposes. Solid state fermentation of four substrates (sawdust of *Anthostema aubryanum* Baill, *Mansonia altissima* (A. Chev.) A. Chev., *Nauclea diderrichii* (De Eild & T. Durand) Merr and Malt Extract Agar) contaminated with various levels of crude oil contamination by *Leucocoprinus cretaceus* (Bull.) Locq., was studied. The effect of crude oil on mycelial biomass production, polysaccharide, and polyphenol contents of *L. cretaceus* during solid state fermentation of lignocellulosic wastes was determined. The polysaccharide and polyphenol content of the mycelia was determined with the Phenol-Sulphuric acid and Folin-Ciocalteu methods, respectively. Solid state fermentation of crude contaminated substrates improved biomass and polysaccharide content of *L. cretaceus*. The fungus is a good bioremediation agent degrading crude oil with increasing levels of crude oil contamination (28.00% in 1.00% crude oil contamination to 81% in 10.00% crude oil contamination). Radial mycelial extension increased with increasing levels of crude oil contamination. Crude oil did not cause increased polyphenol concentration and therefore not a stress factor. There was increase in polysaccharide content indicating metabolization of crude oil for metabolic build up. Supplementing crude oil contaminated substrates with the sawdust of *M. altissima* resulted in the highest level of crude oil degradation by the test fungus. *L. cretaceus* is a potential strong myco-remediation agent. This study records the first time the fungus is used for degradation of crude oil contaminated substrates. The mushroom has the potential to completely mineralize petroleum hydrocarbons.

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Nigeria is a major producer of crude oil faced with problems that come with exploitation of crude oil and its products for financial benefits. The major problem that come with the crude oil industry is contamination of the environment. The clean-up of contaminated sites is a priority to the nation. The clean-up of the environment can be achieved by applying chemical, physical thermal and biological treatments. The biological treatment is however preferred to the other treatment options. One of the biological agents used for clean-up of crude oil contaminated sites are white rot fungi. It is important that indigenous plants, fungi, algae, and bacteria are assessed as bioremediation agents as they have acclimatized to the environmental conditions in Nigeria. White rot fungi abound in the wild in Nigeria and their use have not been fully exploited. Mushrooms are organisms that assemble their food from the environment by secreting extracellular digestive enzymes, decomposing complex food materials found in organic biomass from which they produce simpler compounds to build up their body mass (Verma, *et al.*, 2013). *Leucocoprinus cretaceus* is a white rot fungus characterized by lepiotoid habit and has a pantropical distribution. *L. cretaceus* are found in greenhouses in temperate regions as foreign species (Niveiro, *et al.*, 2012). The mushroom can be found on leaf litters in fallow plots and green houses. The fungus can produce laccase and cause dye discoloration like other white rot fungi (Jebapriya and Gnanadoss, 2014). The ability to produce laccases makes *Leucocoprinus cretaceus* a potential myco-remediation agent. The fungus has not been previously studied for decontamination of crude contaminated substrates. White rot fungi have laccases which are enzymes capable of degrading organic pollutants (Bodke, *et al.*, 2012). Degradation of crude oil and polyaromatic hydrocarbons like fluoranthene by white rot fungi are linked to biomass production and utilization of the laccase enzyme (Wirasnita and Hadibarata, 2016). The ability to convert crude oil to biomass is a good indication the white rot fungus is a potential candidate for crude oil spill clean-up. There are other enzymes linked to degradation of organic contaminants such as peroxidases, dioxygenases and ligninases which are potentially present in *Leucocoprinus* species (Okino-Delgado, *et al.*, 2019).

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Secondary metabolites such as naphthalene-1, 8-dicarboxylic acid and phthalic acid are linked with the degradation of polyaromatic hydrocarbons like fluoranthene by white rot fungi (Wirasnita and Hadibarata, 2016). Decontamination of crude oil in soil by white rot fungi can be enhanced by adding lignocellulosic wastes (sawdust, straw, palm fruit fibre) (Rhodes, 2014). There is direct link between degradation of petroleum hydrocarbon-based contamination and biomass formation (Bodke, *et al*., 2012). The type of substrate and the species of mushroom involved determines the degree of degradation attained. It is important the right substrate combination is sought for complete mineralization of the contaminant. Specific fungi choose specific lignocellulosic wastes for their growth. The current investigation seeks to know the type of substrate combination that will enhance the degradation process. The use of lignocellulosic wastes for cultivation of mushrooms for food, drugs and other by-products is sustainable means of ensuring that we have a circular economy where natural resources are used in a wise and environmentally friendly way for economic and environmental restoration. The sawdust of wood found commonly in sawmills are investigated for use in bio-stimulation of crude oil polluted substrates, turning waste to useful products. The sawdust of *Anthostema aubryanum* Baill, *Mansonia altissima* (A. Chev.) A. Chev., *Nauclea diderrichii* (De Eild & T. Durand) Merr were used for this study. *A. aubryanum* is a member of the family Euphorbiaceae and native to Benin, Cabinda, Congo, Ghana, Gulf of Guinea, Ivory Coast and South Nigeria. It is known as Odogbo in Nigeria with medicinal properties like diuretic and astringent according to the Kew Botanic Garden data base. It is a soft wood, good as firewood with latex in all its parts which can cause blindness. It is known medicinal plant in Benin Nigeria. The bark is used as fish poison in Gabon. The wood is not commonly used because, it is extremely abrasive in sawing but holds nails very well, not durable but has little resistance to fungi (Oyen andLouppe, 2011). *Mansonia altissima* (A. Chev.) A. Chev., is a member of the family Sterculiaceae (APG: Malvaceae) and native to West tropical Africa-Guinea, Cote D'Ivoire to the Central African Republic and Northern DR Congo. It is hard-wood and has medicinal properties (used for treatment of leprosy, scabies, yaws, and syphilis. It finds use in general and high-class joinery, cabinet work, furniture, decorative veneer, and handicrafts. It is particularly useful in construction of doors and windows. Large quantity of sawdust is therefore generated from the use of the wood. The bark is poisonous and should be avoided and sawdust are mostly discarded and not used for other products. Its use here is extremely useful as the sawdust not desirable for other uses can be used to potentially enhance crude oil spill clean-up by a white rot fungus. The wood waste has been used for the cultivation of *Pleurotus tuber-regium* an indigenous white rot fungus. *N. diderrichii* wood is of high value in the building industries in Nigeria and therefore has been over-exploited. A large quantity of wastes is therefore generated from the use of this plant. The sawdust generated can be harnessed potentially in the environmental restoration of the environment from crude oil spills. It is a dense wood belonging to the family Rubiaceae and resistant to termites and therefore difficult to degrade. White rot fungi however can degrade this wood harnessing it for biomass production. The plant is also considered a medicinal plant by locals across West Africa (Haudecoeur, et al., 2018). The production of polyphenols is a stress response factor, and this is also investigated to find out the degree of stress that crude oil will impact on the test fungus *L. cretaceus.*

MATERIALS AND METHODS

Collection and identification of specimens: Fruiting bodies were collected from a fallow plot near the Center for information technology System (CITS) building (GPS-N 006° 31' 90" E 003° 24' 20") at the University of Lagos. The mushroom was identified using microscopic and macroscopic features with identification keys in standard manuals (Largent, 1986).

Tissue culture initiation of L. cretaceus and Spawn preparation: Sterile Tissues from fresh sporophores of *L. cretaceus* were excised aseptically onto modified malt extract agar medium in Petri plates and incubated for 7-10days. Primary mycelium from agar plates were used to produce spawn on sterilized *Sorghum bicolor* grains following a modification of methods by Mleczek, et al., (2018). Grain spawn was used as mushroom 'seed' for the fermentation studies.

Solid-State Fermentation Design: Four types of substrates were used in the experiments viz- sawdust of locally available hardwoods *A. aubryanum, M, altissima, N. diderrichii* collected from sawmill at Okobaba, Yaba LCDA Lagos State, Nigeria. Malt extract agar was the fourth substrate used. All substrates were additionally supplemented with 1% gypsum, 0.02% KH_2PO_4 and 0.01% $MgSO_4$. The doses of the supplements are expressed as percentage of the weight of the substrates. Two hundred (200g) of the substrates were weighed into jam jars. All substrates except control A were contaminated with five levels of crude oil contamination (1.00, 2.50, 5.00, 7.5 and 10.00%). Control A had no crude and control B had crude oil but was not seeded with the fungus.

All treatments had five replicates each. The substrates were sterilized at a temperature of 121^0C for 1hour and cooled down to room temperature. The substrates were inoculated with 10g of the *Sorghum bicolor* grain spawn and incubated in semi-aerobic conditions. They were incubated in a DNP 9082 laboratory incubator at 30 °C in static condition for 40 days at a relative humidity of 80-85% until substrates were completely covered with mycelium. The initial weight of the substrates was noted, and final weights also recorded.

Quantification of mycelial biomass in solid substrates: The whole solid substrate/media were dried in an oven at 100^0C until constant weight was obtained and pulverized. The mycelia biomass in the dry solid media was estimated by quantifying the structural chitin component, the N-acetyl-D-glucosamine (NAGA) released after hydrolysis with 6 N HCl adopting methods by (Plassard, Mousain, & Salsac, 1982). The analytical grade NAGA was used as reference. A separate experiment in broth medium was conducted to get data for estimation of NAGA content in the mycelium. The parallel experiment was run in a 250ml conical flask in a 100ml liquid broth with the following composition – (g/L): 30g glucose, 6g of yeast extract, 0.5g of $MgSO_4.5H_2O$, and 0.1g $CaCl_2$. The NAGA content of the mycelium grown in the broth medium was determined also by adopting methods by (Plassard, *et al*., 1982). The obtained conversion factor for the test mushroom- *L. cretaceus* was 145.38.

Determination of Total Polysaccharide Content: Polysaccharides were extracted following modification of methods by Montoya, et al., (2020). Dry pulverized biomass (100mg) was mixed with 5ml of concentrated analytical grade ethanol and left in an ultrasound-sonicator for 20minutes, removed and left to soak for 24hrs. The extract was filtered with pre-weighed glass microfiber glass filter (Whatman GF/F) and centrifuged for 30minutes at 10,000 rpm. The precipitate was resuspended in 1-M sodium hydroxide solution, left to incubate at 60^0C for 1 h. Total polysaccharide content of the test mushroom grown in crude oil contaminated substrates was evaluated using phenol-sulphuric acid test as described by (Dubois, Gilles, et al., 1956). In this protocol 0.05 ml of 80 % phenol is added to 2 ml of mushroom extracts (polysaccharide), rapidly followed by addition of 5 ml concentrated sulphuric acid (95 %). The mixture was allowed to react for 20 minutes, and absorbance read at 490 nm in a spectrophotometer. Standard curve readings were used to extrapolate concentrations of the polysaccharide content of the extracts. The total polysaccharide content was expressed as mg per g dry solid.

Determination of Total Polyphenol Content: Total polyphenolic content was determined for test mushroom biomass extract (methanol extract) with the Folin-Ciocalteu method (Slinkard and Singleton, 1977). The absorbance was measured with a UV Spectrophotometer (Shimazu, UV-1800) at 765 nm against a methanol blank. The analysis of total polyphenols was performed in triplicate. The outcome data were expressed in mg/g of gallic acid equivalents in milligrams per gram (mg GAE/g) of dry extract.

Extraction and Gravimetric Determination of Degraded Crude Oil: Determination of degradation efficiency of *L. cretaceus* was estimated by adopting modification of methods by Al-Hawash, et al., (2018). Residual crude oil was extracted from substrates (10g) and control substrates (crude oil B-no mushroom) with analytical grade chloroform (100ml). The chloroform was evaporated at 55^0C in a water bath with a rotary evaporator. The residual crude oil was dried over anhydrous sodium sulphate. Gravimetric Determination of residual crude oil after biodegradation in the various contaminated substrates was achieved by weighing the quantity of crude oil before and after biodegradation. The estimated crude oil degradation efficiency was calculated following the formula Known weights (10g) are taken from all treatments (crude oil contaminated substrates and control A)

Percentage degradation was calculated as follows:

$$A = B - C$$

Where A = weight of residual crude oil; B = weight of beaker with extracted crude; C = weight of empty beaker

$$D = E - A$$

Where D = amount of crude oil degraded; E = weight of crude oil added to the substrate; A = weight of residual crude oil

$$\%\ DF = \frac{D}{E}\ x\ 100$$

Where DF = degradation efficiency; D = amount of crude oil degraded; E = weight of crude oil added to the substrate.

Statistical analysis: Results are expressed as means with standard deviation calculated using statistical package for social science (SPSS) and a one-way analysis of variance (ANOVA) was performed. The

Duncan's multiple range test was used to identify significant differences among treatments $p \geq 0.05$.

RESULTS AND DISCUSSION

Crude oil contamination did not affect the growth of the test mushroom *L. cretaceus* negatively (Plate 1). The mushroom grew in all tested levels of crude oil contamination (1.00-10.00% crude oil contamination) (Plate 2). Higher levels of crude oil contamination resulted in higher radial extension (Plate 2, Figure 1)

Plate 1: Test Mushroom- *Leucocoprinus cretaceus* (Bull.) Locq

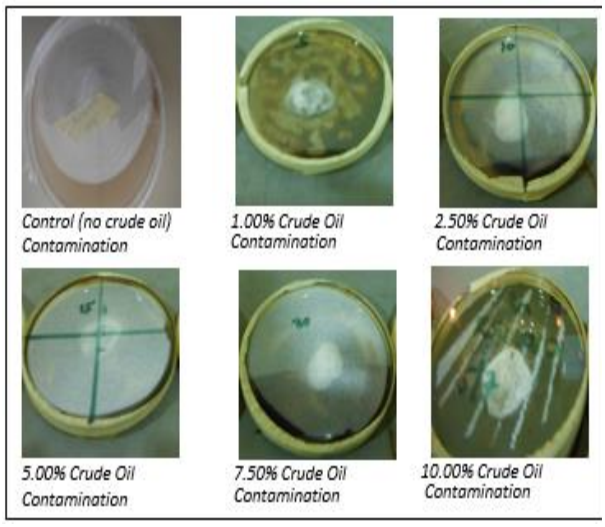

Plate 2: Efffect of Various Levels of Crude Oil Contamination on Mycelia Radial Growth of *L. cretaceus*

There was no significant difference between control (no crude oil) and 10.00 level of crude oil contamination. Effect of crude oil contamination on radial extension of *L. cretaceus* varied from 7.60cm in 7.50% level of crude oil contamination to 8.86cm in control (no crude oil contamination). Slight inhibitory effect was noticed at 7.5% level of crude oil contamination from days 7 to 10 (Figure 1). *L. cretaceus* degraded/metabolised crude oil performing better at higher levels of crude oil contamination. Supplementation with lignocellulosic wastes enhanced the degradative potentials of the mushroom. The stimulatory effect of the lignocellusic wastes varied with *M. altissima* been the best. The degradation efficiency increased from 28.00% (Malt extract agar substrate) in 1.00% level of crude oil contamination to 92.77% (*M. altissima* substrate) in 10.00% level of crude oil contamination. In control B treatments crude oil loss was due to abiotic factors, it however varied from 1.08% in Malt Extract Agar substrate to 8.55% in *M. altissima* (Figure 2). Crude oil contamination had a stimulatory effect on biomass formation by the mushroom *L. cretaceus* (Figure 3). The stimulatory effect was not affected by the levels of crude oil contamination with the various crude oil contaminated substrates (Figure 3). Biomass production varied from 10.30mg/g dry solids in Malt extract agar substrate (Control A-no crude oil treatment) to 226.78mg/g dry solids in 2.5% crude oil contaminated *N. diderrichii* substrate (Figure 3). Crude oil contamination at all tested levels did not affect polysaccharide content of mycelia biomass produced from *A. aubryanum*, *M. altissima* and *N. diderrichii* instead causing a stimulatory effect. The least polysaccharide content was recorded in biomass from crude oil contaminated malt extract agar substrates at 10.00% level of crude oil contamination (Figure 4). The malt extract agar substrate had no lignocellulosic waste but was not adversely affect and recorded the least polysaccharide content. Polysaccharides are formed as primary metabolites in mushrooms as the results here indicate that there was higher cell growth rate as polysaccharides are formed during colonization of the substrates (Tang and Zhong, 2002; Sanchez and Montoya, 2020). The polyphenol content of *L. cretaceus* varied with the substrate type and level of crude oil contamination. There was reduction in the polyphenol content of the *M. altissima* substrates that had the highest biomass and polysaccharide content. This study concludes as already in stated in literature that polyphenols are secondary metabolites not affected by normal cell growth activities or metabolism. The substrate *A. aubryanum* however improved the polyphenol content of the mushroom indicating that the wood can be used to produce high quality biomass when considering producing biomass for pharmaceutical purposes. The polyphenol content of the mushroom therefore varies with the medium or substrate composition and

mushroom species as reported also by Dulay, et al., (2015).

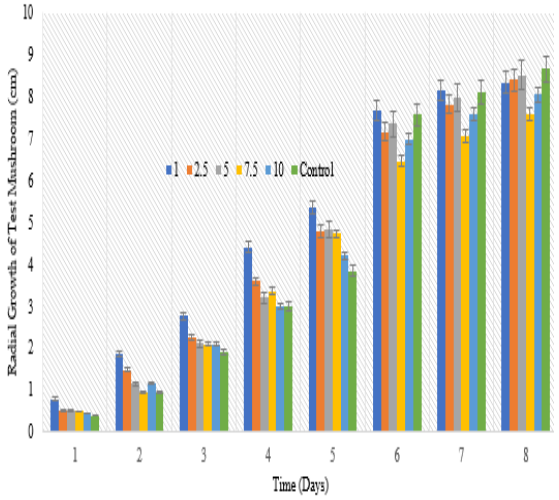

Fig 1: Effect of Crude Oil on Radial Growth of L. cretaceus in Various Levels of Crude Oil Contamination in Malt Extract Agar Medium

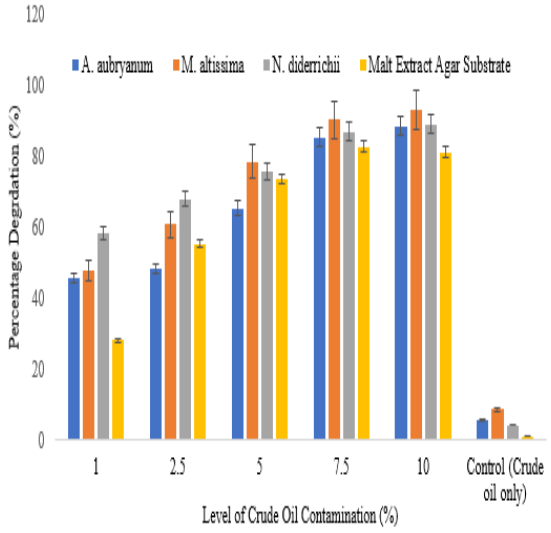

Fig 2: Degradation Potential of *L. cretaceus* in Various Levels of Crude Oil Contaminated Substrates

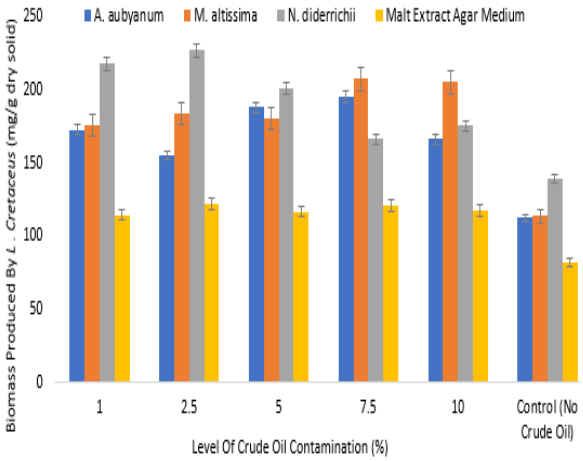

Fig 3: Effect of Various Levels of Crude Oil Contaminated Substrates on Biomass Production by *L. cretaceus*

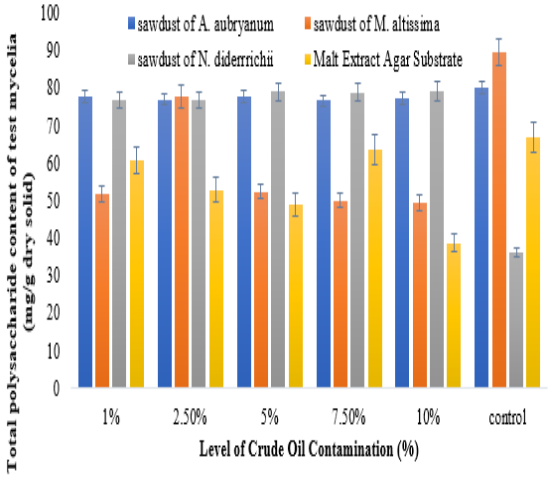

Fig 4: Effect of Diverse Levels of Crude Oil Contaminated Substrates on Total Polysaccharide Content of *L. cretaceus* mycelia Biomass

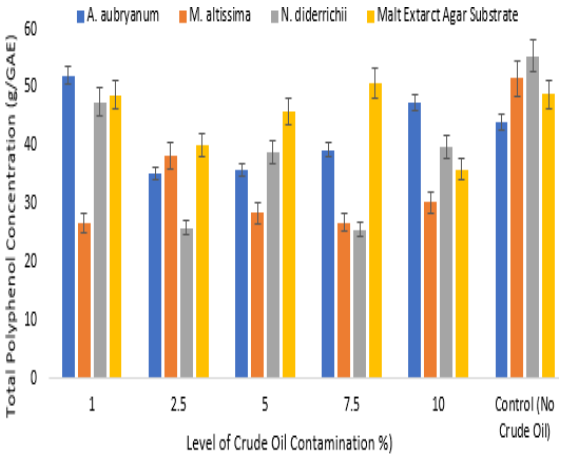

Fig 5: Total polyphenol content of *L. cretaceus* mycelia biomass harvested from diverse levels of crude oil contaminated lignocellulosic waste

The stimulatory effect reported here in this study for *L. cretaceus* had been previous recorded that petroleum hydrocarbon improved the growth of white rot fungi in contaminated soil by increasing their size and yield indicating that the pollutant has a fertilizer effect (Stamets, 2005). Production of the mycelium biomass and polysaccharides by *L. cretaceus*, in solid state depended on the growth medium -wood sawdust used (Avni, et al., 2017). This agrees with work done by Verma, et al., (2013) where production of mycelia biomass and polysaccharides by *P. ostreatus*, in submerged culture, depended on type of substrate and composition. The current study also agrees with the work of Reverberi, et al., (2004) because olive oil mill solid waste treated with *Pleurotus eryngii* after supplementing with sawdust of *Eucalyptus* recorded increased biomass and polysaccharide content. There was increase in polysaccharide content due to the reaction of the mushroom to oxidative stress caused by the olive oil according to Reverberi, et al., (2004). The white rot fungus was able to mineralize crude oil and the lignocellulosic wastes as shown by increase in biomass formed in the crude oil contaminated substrates. Supplementing with the sawdust of hardwoods enhances the degradative potential of the white rot fungi. The phenolic content varied with the substrate composition which agrees with the work of Gaitan-Hernandez, *et al*., (2020). There was no increase in phenolic content compared to control A (no crude oil contamination). The stress factor induced by the presence of crude oil was well tolerated by the test mushroom.

Conclusion: The current study shows that utilization of inexpensive lignocellulosic residues like sawdust in crude oil contaminated substrate has a stimulatory effect on crude oil degradation causing a near complete removal of crude oil. The addition lignocellulosic wastes enhanced production of high value mycelia biomass with high polysaccharide content. The experiment concludes that polysaccharides were primary metabolites. It is the first time that *L. cretaceus* is used for myco-remediation of crude oil contaminated substrates and concludes that the mushroom is an exceptionally good bioremediation agent. Solid state fermentation of lignocellulosic residue is a promising and cost-efficient way of producing polysaccharides from white rot fungi during the mycelia/ colonization stage. Sawdust of *Mansonia altissima* performed well and should be considered for improved mushroom growth with regards to polysaccharide and polyphenol contents of the mushroom. Sawdust of hard or soft wood enhances crude oil degradation by this fungus.

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