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EFFECT OF VARIOUS GROWTH CONDITIONS ON PHENOL DEGRADATION BY TWO *Bacillus cereus* STRAINS

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ABSTRACT

Phenol is a toxic environmental pollutant. Two strains of *Bacillus cereus* (RC29 and RM4) capable of utilizing phenol as their carbon source were selected to study the effects of various growth parameters such as pH, temperature, volume of inoculum, period of incubation, and the concentration of substrate (phenol) on its phenol degradation abilities and thereby optimizing the growth conditions for their maximum phenol degradation. The minimal salt phenol medium (MSPM) was used. Both the strains gave maximum phenol degradation under ideal growth conditions of pH 7, temperature 37^{0}C, 5% (v/v) inoculum volume, and an incubation period of three days. At high substrate concentration, the growth and degradation were found to be decreasing gradually. For strain RM4, the highest degradation was perceived at 40 mM phenol concentration (39.47%). For RC29, it was observed to be 60mM concentration (39.22%). It was also observed that there is a strong relationship between the volume of inoculum and the rate of phenol degradation. The enhanced phenol-degrading ability of these strains under the optimized conditions can be suggested as a promising tool for the treatment of phenol-contaminated wastewater or effluents.

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INTRODUCTION

Phenolic compounds are widespread in the environment. They are formed naturally during the decomposition of various substances of plant and animal origin, mainly polymers containing aromatic rings. Phenolic acids, such as O-coumaric acid, caffeic acid, ferulic acid, P-coumaric acid, P-hydroxy benzoic, syringic acids, and vanillic acids, are commonly found in plant tissues and the soil in which the plants are cultivated. Phenolic compounds are the typical by-products of any industrial process, such as the manufacture of dye and dye intermediate industries, plastics, drugs, explosives, antioxidants, paper and petroleum industries, and rubber production and processing industries. Phenol itself is an established disinfectant and germicide used by household cleaners. These are discharged along with the effluents from several categories of industries such as textiles, woollen mills, coke ovens, pulp and paper industries, iron and steel plants, petrochemicals, paint industries, resins, cosmetics, oil-drilling and gas extraction units, pharmaceuticals, coal washeries, refractory industries, etc., and enter into various environmental matrices. Phenols may be released into the soil or sediments during the manufacturing process when spills occur during loading, transport, and leaching from hazardous waste sites and landfills. Phenols that leach through the soil to groundwater spend at least some time in that soil as they travel to groundwater. Organic contamination prompted by high organic content in aquatic ecosystems and eutrophication are the two sorts of substantial and long-lasting pollution dangers of water bodies. Organic pollutants are derived from farm water, industrial effluents, urban run-off, and domestic sewage (Muthanna & Afaf, 2018). Phenols are toxic, carcinogenic, mutagenic, and teratogenic. Unfortunately, phenols and phenolic compounds have now become common environmental pollutants found in potable water, soil or sediments, and ambient air. Eleven phenolic compounds are included in the list by the United States Environmental Protection Agency (USEPA, 2014) and European Commission (EC) as priority pollutants. Other national-level regulatory bodies, such as the Ministry of Environment and Forests (MoEF) and the Central Pollution Control Board (CPCB) under the Government of India, also listed phenols and phenolic compounds as the priority pollutants. In India, the regulatory actions for phenols and phenolic compounds were contemplated under the Environmental (Protection) Rules, 1986, under which several environmental standards for the discharge of phenols and phenolic compounds in industrial effluent have already been notified. Many phenolic compounds are subjected to regulations

for air and water pollutants around the world. Some are persistent in the environment. Industrial waste carrying different types of phenolic compounds may transform the normal vegetation, which finally leads to the destruction of the native flora and fauna and thereby imbalances the ecosystem. The accumulation of these toxic chemicals may create mutations in the genes of native species. It may also change the physiochemical and biological properties of the biotic and abiotic systems (soil, water). Removal of phenols from the environment improves the quality of natural vegetation. Therefore, it is essential to control and monitor the use of phenols and their proper treatments. Many microbes are involved in the toleration and degradation of phenols and phenolic compounds. It was identified that the enzyme system of several species of microbes, including algae, fungi, and bacteria, is actively involved in the degradation of phenols. More studies are to be conducted in this area to discover new species or strains causing degradation. Those species can be developed to effectively treat the phenols released into soil and water for a better life. Modern environmental biotechnology, together with microbial enzymatic systems for the effective degradation of phenols, can make a dramatic change in the treatment of environmental pollutants like phenols. A lot of microorganisms were found to be tolerant to phenol. Microbial processes can be used for environmental protection and improvement. Bio-separations and bioreactor technologies help in the process of environmental cleaning. Environmental and economic benefits in manufacturing, monitoring, and waste management are offered by the field of environmental biotechnology. Treatment methods such as activated carbon adsorption, ion exchange, chemical oxidation, liquid-liquid extraction, etc., are effective, but they often suffer from drawbacks such as high cost. Hence, biodegradation is considered an environment-friendly and cost-effective alternative method.

Phenol biodegradation in soil may take place under both aerobic and anaerobic conditions. Microorganisms (bacteria, fungi, yeasts, and algae) use phenols as the sole carbon source for the growth of the organisms. Most of the cases are reported from bacterial species. The degradation process was catalyzed by a variety of enzymes such as oxygenases, hydroxylases, peroxidases, oxidases, laccases, and tyrosinases (Nair *et al*., 2008). Degradation under aerobic conditions is initiated by oxygenation. The aromatic ring is initially monohydroxylated by a mono-oxygenase phenoldehydroxylase at an ortho position to the pre-existing hydroxyl group to form catechol. This includes *Pseudomonas, Bacillus, Acinetobacter, Streptomyces, Desulfobacterium, and Geobacter*. Anaerobic degradation of phenol is less advanced than the aerobic process. The first step in this pathway is the carboxylation of phenol at the para position to 4-hydroxybenzoate by the enzyme 4-hydroxybenzoate carboxylase. *Thauera aromatica* K172 and *Clostridium species* are examples of anaerobic degradation of phenols (Schie & Young, 2000). Klekner & Kosaric, 1992, investigated the fact that 2,4-dimethylphenol was converted to an isomer of dimethylbenzenediol by a species of *Chlorella*. It was also capable of degradation and dechlorination of 2-chlorophenol. *Scenedesmus specie*s, after an adaptation of a period of 5 days, showed quick degradation of 2,4-dinitrophenol at a concentration of about 190 $mg1^{-1}$. All algae tested by Klekner & Kosaric in 1992 for their study have a mechanism for the degradation of phenolic compounds.

Many *Bacillus species* have been identified as potent phenol-degrading strains, such as *Bacillus stearothermophilus, B. laterosporus, B. brevis,* and *B. thermoglucosidasius* (Krastanov *et al*., 2012). *Bacillus subtilis sp*3, a PHB-producing bacteria, was found to degrade phenols, which can also be used for the laboratory-scale treatment of paper mill effluent containing phenolic compounds (Nair & Prakash, 2017). Two strains of *Bacillus cereus* (*B. cereus* MTCC 9817 and 9818) isolated from a petroleum refinery and oil exploration site showed high tolerance to phenol at a concentration of 1000 mg/L, under an optimum temperature of 37^0C and p^H 7.0 (Banerjee & Ghoshal, 2010). At an optimum p^H of 8.0, temperature 34^0C, 5% (v/v) of inoculum size, and without any co-substrate, a strain of *B. brevis* degraded 1750 mg/L phenol in 144 hours (Arutchelvan *et al*., 2006). *Sulfobacillus acidophilus* isolated from hydrothermal vents in the Pacific Ocean has a maximum degradation rate of 2.32 mg/L/h of phenol at 38 hours. The degradation is aerobic via the meta-pathway under p^H 1.8 and 45^0C temperature. Another strain, *S. acidophilus,* can tolerate 1300 mg/L phenol and completely degrade 100 mg/L phenol in 40 hours (Zhou *et al*., 2016). The peroxidase enzyme isolated and purified from *B. aryabhattai* B8W22 exhibits the highest degradation (98.47%) at p^H 5.8 with maximum activity at 30^0C and complete thermal stability at 40^0C. The enzyme showed 0.012 U/mg specific activity (Elmetwalli *et al.,* 2023). *Bacillus badius* follows ortho and meta-catechol pathways. At high concentrations of substrate (phenol), the rate of degradation by *B*. *badius* was more than 70%, whereas 98% was degraded at lower concentrations with an alkaline p^H of 9.0 at 37^0C (Sarwade & Gawai, 2014). However, the process degradation depends upon the optimal conditions of p^H, temperature, concentration of substrate, volume of inoculum, days of incubation, speed of rotation of the shaking incubator, etc. The p^H value of the medium influences the production of degrading enzymes and finally affects the growth and metabolism of the microbe. An optimum temperature is required for the activity of degrading enzymes (Zhang *et al*., 2022). The duration of incubation and the concentration of available substrate also have a crucial role in the biodegradation of phenols. In this study, two strains of *Bacillus cereus* species, RM4 and RC29, were used to monitor their efficacy in phenol removal. The main purpose of this study is to identify the optimum conditions required for maximum phenol degradation by the strains.

MATERIALS AND METHODS

In this study, soil samples from six different areas of the selected site (an industrial area in the Kottayam district of Kerala, India) were collected, and the pooling method was adopted. Bacterial colonies capable of phenol degradation were isolated from the soil by the continuous-enrichment culture method. The soil samples were then serially diluted into agar plates containing low concentrations of phenol (10 mM). The colonies that showed growth in the medium were selected and then transferred to the next higher concentration of phenol- containing nutrient agar and broth. This process was continued with successive concentrations of phenol until no growth in the medium and broth was obtained. The colonies that showed growth in both agar plates and broth culture were selected and screened for their maximum phenol degradation capacity. For this, the minimal salt medium containing various concentrations of phenol (MSPM) of p^H 7 was used (Nair I, C., 2017). 100 ml of the MSPM inoculated with each strain were incubated in a shaking incubator at 150 rpm speed and 37^0C temperature for five days. The rate of degradation exhibited by each isolated strain at different concentrations of phenol ranging from 20, 20, 40, 60, 80, and 100 mM was studied. Finally, two bacterial strains named RM4 and RC29 showed maximum degradation at various concentrations of phenol and were isolated. The two strains were then identified through, morphological and biochemical as well as molecular characteristics (16srRNA sequencing). The strains were identified as *Bacillus cereus* strains. In this study, the cultural conditions were optimized for the two strains to obtain maximum degradation. For this, the following materials and methods were adopted.

Materials Used: All the chemicals used for the experiment are from HIMEDIA and Nice Chemicals. The glassware used is manufactured by Borosil and Nice. Materials used include polythene covers, sterile loops, test tubes and racks, measuring cylinders, conical flasks, standard flasks, micropipettes, sterile tips, petri plates, non absorbant cotton, and various growth media. The equipment and instruments used include a shaking incubator, a laminar air flow chamber, an incubator, a pH meter, a weighing balance, a hot air oven, an autoclave, microscopes, and a spectrophotometer.

Estimation of phenol: Estimation of phenol was done using the 4-aminoantipyrine method (4-AAP method, APHA, 1978). Aminoantipyrine reacts with phenol in the presence of potassium

ferricyanide to give a red-colored compound called antipyrine, whose absorbance was recorded at 500 nm. The concentration of this dye is directly proportional to the concentration of phenol in the sample. Different aliquots of the stock phenol solution were prepared, and the assay was carried out. The readings were recorded, and a standard graph was prepared.

Calculation of Percentage Phenol degradation: The initial and final concentrations of phenol in the samples (MSPM with inoculum) and control (MSPM without inoculum) were measured using the 4-AAP assay. The percentage of degradation was calculated using the following equation from the standard graph prepared:

$$\text{Percentage phenol-degradation} = \frac{C_i - C_f}{C_i} \times 100$$

Where, C_i = initial concentration of phenol in the medium.
C_f = final concentration of phenol in the medium.

The concentration of phenol in the medium was expressed in millimolar (mM) solution.

Standardization of Inoculum: 1 ml of each bacterial inoculum was added to 500 ml of nutrient broth medium containing 10 mM phenol. The inoculated medium was then incubated overnight at 37^0C temperature and 150 rpm speed in an orbital shaking incubator. The broth containing bacterial cells was then centrifuged at 2000g for eight minutes. The pellet settled down was washed using an 86% sodium chloride solution. The collected cells were then diluted with the same sodium chloride solution until the OD_{500} becomes one. This solution is used as the standard inoculum for the study.

Optimization of p^H : 5% (v/v) of the standard bacterial inoculum was added to a 250 ml conical flask containing 100 ml, 10mM MSPM with different p^H values. A p^H value ranging from 5.0 - 9.0 with an interval of one was selected for the study. The initial p^H of the MSPM was adjusted using 1N NaOH or HCl. The medium without inoculum is used as the control. The samples, along with the control, were then kept in a shaking incubator at 150 rpm and 37^0C temperature for three days. The OD values were recorded, and the percentage of phenol degradation was calculated. The p^H value at which the strain exhibits maximum phenol degradation was selected as the optimum p^H value for maximum phenol degradation.

Optimization of temperature: The temperatures selected for the study are 27^0C, 32^0C, 37^0C, 42^0C, and 47^0C. 100 ml of 10mM, MSPM (pH 7) was inoculated with 5% (v/v) of each bacterial inoculum and incubated in a shaking incubator for three days at 150 rpm speed. The OD values of the samples along with the control were recorded, and the phenolic content was estimated. The percentage of phenolic degradation was calculated and analyzed. The temperature at which maximum degradation occurred was regarded as the optimum temperature.

Optimization of concentration of substrate : Phenol is the only source of carbon present in the MSPM. The percentage of phenol-degradation at various concentrations of substrate (phenol) was studied by inoculating 5% (v/v) of each bacterial inoculum into 100 ml of MSPM of pH 7 and incubating at 37^0C and 150 rpm speed in a shaking incubator for three days. The concentration of phenol taken for the study ranges from 10 mM, 20, 40, 60, 80, 100, 120, and 140 mM.

Optimization of volume of inoculum : Different volumes (% v/v) of bacterial inoculum were taken for the study, such as 1%, 2%, 3%, 4%, and 5%. 100 ml of 10 mM, MSPM of pH 7 was inoculated with different volumes of inoculum, and the medium without inoculum was kept as the control. The samples were incubated at 37^0C for three days in a shaking incubator at 150 rpm speed. The volume of inoculum that gives maximum degradation was regarded as the optimum.

Optimization of incubation period: 100 ml of 10 mM MSPM was inoculated with 5% (v/v) of inoculum and incubated in a shaking incubator at 150 rpm speed and 37^0C temperature for five days. The percentage degradation was recorded after one (24 hrs), two (48 hrs), three (72 hrs), five (120 hrs), and seven days (168 hrs), respectively. The incubation time at which the maximum percentage of phenol degradation obtained was regarded as the optimum incubation period. Each study was conducted three times.

RESULTS AND DISCUSSION

Estimation of phenol: Estimation was done by 4-AAP assay. A standard graph was plotted with the concentration of phenol on the X-axis and absorbance values measured at OD_{500} on the Y-axis. More or less, a straight line was obtained. From the standard graph, the initial and final concentrations of phenol in the control and samples were estimated to calculate the percentage of phenol degradation exhibited by each *Bacillus* strain.

Optimization of pH: pH has a significant role in degrading phenol. The optimum pH for the effective degradation of phenol by both the strains was found to be 7. RM4 and RC29 showed 34.94% and 36.73% degradation (Table 1). For most Bacillus species, the optimum pH for growth and degradation was identified to be around pH 7 (Banerjee & Ghoshal, 2011; Khleifat & Khaled, 2007; Nair & Prakash, 2017; Mohammad *et al.*, 2021). Two strains of *Bacillus cereus*, AKG1 and AKG2, were reported by Banerjee & Ghoshal (2010). The result was also supported by the findings of Nair & Prakash in 2017. They identified a PHB- producing *B. subtilis* sp3 capable of maximum growth and phenol degradation at pH 7. The best pH for growth and degradation by *B. simplex* was reported to be pH 7 by Mohammad *et al.* in 2021. From the study, it was obvious that the growth and degradation decrease at very low and high pH values. The initial pH value of the medium will affect the synthesis of microbial enzymes and their catalytic activity, which further affects the growth of microorganisms and their ability to degrade pollutants (Zhang *et al*., 2022). Too high or too low pH values often lead to the inactivation of most degrading enzymes of the bacteria and thereby inhibit the degradation of phenol.

Table 1. Table showing pH optimized for phenol degradation by RM4 and RC29

Sl.no	pH value	Phenol degradation (in %)	
		RM4	RC29
1	5	22.38 ± 0.45	18.21 ± 0.36
2	6	25.16 ± 0.50	29.64 ± 0.60
3	7	34.94 ± 0.70	36.73 ± 0.73
4	8	27.81 ± 0.56	31.33 ± 0.63
5	9	20.14 ± 0.40	23.56 ± 0.47

"±" indicates standard deviation

Optimization of temperature: Another important factor that affects the ability of a microbe to degrade an organic substance is the temperature. In this study, a temperature of 37^0C was determined to be the optimum temperature for maximum phenol degradation (Table 2). Both the strains attained maximum phenol removal at 37^0C (34.38% for RM4 and 36.09% for RC29). Studies on *Bacillus species* have shown that temperature plays a vital role in phenol degradation, with the optimum temperature of 37^0C (Banerjee & Ghoshal, 2010; Farag *et al*., 2021; Zhang *et al*., 2022). At a higher temperature, the activity of the degrading enzyme may get inactivated. It was also reported that, at very low temperatures, the activity of enzymes decreases and thereby results in slow microbial metabolism. Farag *et al.* in 2021 isolated a marine *Bacillus subtilis* AAK capable of degrading six diverse phenolic compounds, of which 2,4-DCP was effectively degraded at an optimum temperature of 37^0C. Zhang *et al.* (2022) reported that an optimum temperature of 37^0C is suitable for efficient phenol degradation and maximum cell growth by the *Bacillus cereus* ZWB3 strain. It is also the same for *B. badius* (Sarwade & Gawai, 2014) and 34^0C for *B. brevis* (Arutchelvan *et al.,* 2006).

Table 2. Table showing the effect of temperature on phenol degradation by RM4 and RC29

Sl.no	Temperature (in ^{0}C)	Phenol degradation (in %)	
		RM4	RC29
1	27	16.71 ± 0.33	12.86 ± 0.26
2	32	20.50 ± 0.41	19.55 ± 0.40
3	37	34.38 ± 0.69	36.09 ± 0.72
4	42	31.20 ± 0.62	33.74 ± 0.67
5	47	27.60 ± 0.55	26.00 ± 0.52

A phenomenon has been reported by Meena *et al*. (2016) that if the temperature is too low or too high, the pollutant removal ability of a microorganism becomes deteriorative. All these findings suggest that the ideal temperature for phenol degradation by *Bacillus species* is 37^0C.

Optimization of the period of incubation: The period of incubation is an aspect that controls biodegradation. The minimum period of incubation that promotes maximum phenol degradation is regarded as the optimum incubation period. In this study, an incubation period of three days (72 hrs) was found to be the optimum and was given in Table 3.

Table 3. Table showing the effect of incubation period on phenol degradation by RM4 and RC29

Sl.no	Period of incubation (in days)	Phenol degradation (in %)	
		RM4	RC29
1	One	14.73 ± 0.3	17.44 ± 0.35
2	Two	21.08 ± 0.42	24.00 ± 0.48
3	Three	35.10 ± 0.70	36.83 ± 0.74
4	Five	29.56 ± 0.60	26.54 ± 0.53
5	Seven	17.28 ± 0.35	21.13 ± 0.42

"±" indicates standard deviation

More than 30% of the total phenol was degraded by both the strains in three days. Above this period, the rate of degradation decreases gradually. This result stay close with the findings reported by Nair *et al*. (2008), Long *et al.* (2019), Wen *et al*. (2020), and Gong *et al*. (2021), that the time required for complete degradation of phenol by microbes ranges between 32-75 hrs. Degradation studies conducted in the *Klebsiella oxytoca strain* by Shawabhek *et al.* (2007) found that 75% of the initial phenol concentration of 100 ppm was degraded by the strain within 72 hrs. After 72 hours of incubation, both the strains experience a gradual decrease in their degrading ability. One possible reason for this is that the accumulation of the degradation product in the medium may affect their enzyme activity.

Optimization of volume of inoculums: From most collected data, it was reported that the volume of inoculum has a direct effect on the degradation of phenol by microorganisms. Gong *et al.* (2021) studied the biodegradation of phenol using *Candida tropicalis* SDP-1. He noticed that, a smaller volume of inoculum will extend the lag phase and inhibit the growth of the microbe. In contrast, a high volume of inoculum may lead to intra-specific competition between the microorganisms. In this study, an inoculation volume of 5% was found to be a better choice for the strains RC29 and RM4 (Table 4).

Table 4. Table showing the effect of volume of inoculum on phenol degradation by RM4 and RC29

Sl.no	Volume of inoculum (in % v/v)	Phenol degradation (in %)	
		RM4	RC29
1	1	16.32 ± 0.33	18.10 ± 0.36
2	2	21.84 ± 0.44	23.67 ± 0.47
3	3	26.61 ± 0.53	29.43 ± 0.59
4	4	30.59 ± 0.61	33.88 ± 0.68
5	5	34.96 ± 0.70	36.94 ± 0.74

"±" indicates standard deviation

At 5% volume of initial inoculum, RM4 degraded 34.96% phenol. At the same time, RC29 displayed 36.94% phenol degradation. At low inoculum volume, degradation was found to be deficient. Therefore, it can be concluded that a high volume of inoculum offers more degradation.

Optimization of concentration of substrate: Phenol is the only available substrate used by the microorganism for their growth and metabolism. The growth was found to be negligible on applying a higher concentration of phenol (Sachan & Hussain, 2019). From the study, it was found that, for the strain RM4, a 40 mM phenol concentration was optimum, which gives a degradation rate of 39.47%. The strain RC29 gained a maximum degradation rate at 60 mM phenol concentration (39.22%). High concentration of phenol reduces the ability of the bacterial to degrade phenol (Table 5).

Table 5. Table showing the effect of substrate (phenol) concentration on its degradation by RM4 and RC29

Sl.no	Concentration of substrate (phenol in mM)	Phenol degradation (in %)	
		RM4	RC29
1	20	34.24 ± 0.68	36.82 ± 0.74
2	40	39. 47 ± 0.79	37.61 ± 0.75
3	60	23.65 ± 0.47	39.22 ± 0.78
4	80	19.10 ± 0.38	21.45 ± 0.43
5	100	13.28 ± 0.27	16.41 ± 0.33

"±" indicates standard deviation

This is due to the toxic effect of phenols on microorganisms. Geng & Lim, 2007, stated that a high concentration of phenol can inhibit bacterial growth by reducing the expression of ATP synthase and inhibiting electron transport chain phosphorylation. Due to their toxicity, phenols have an inhibitory effect on cell growth and can cause substrate-level inhibition. These findings were also supported by Yang & Lee, 2007; Paisio *et al.,* 2013; Hussain *et al*., 2015; and Zhang *et al*., 2022.

CONCLUSION

Phenol is a significant pollutant being discharged from the effluents of various industrial sources. Native microbial species in polluted settings were more adaptable than non-indigenous microorganisms, and their dominance aided the bioremediation of phenol-contaminated ecosystems. A critical analysis of the literature reveals that biological treatment is economical, practical, and the most promising and versatile approach, as it leads to the complete mineralization of phenols, producing non-toxic end products (Dankaka & Abdullahi, 2021). The *Bacillus cereus strains* RM4 and RC29 could effectively degrade phenols under optimized conditions of pH, temperature, volume of inoculum, concentration of substrate, and incubation days. Strain RM4 could effectively degrade phenol up to a concentration of 40 mM, pH7, 37^0C temperature, and 5% (v/v) inoculum size. At a substrate concentration of 60 mM phenol, pH7, temperature 37^0C, and an inoculum size of 5% (v/v), the strain RC29 could efficiently degrade phenol. Both the strains could effectively degrade phenol within three days (72 hrs). These results showcase that the strains have the capacity to utilize organic pollutants like phenols as the sole carbon source for their growth and metabolism, suggesting that these strains of *B. cereus* can be applied in the biological treatment of wastewater contaminated with phenol to remove the pollutants under ideal conditions. This study provides findings that are strongly supported by the data reviewed during the course of the study, and can be attributed to the biodegradation studies on phenols.

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Conflict of interest: The authors declare there is no conflict.

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