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Biodegradation of spinetoram (pesticide) by consortium of *Bacillus licheniformis* and *Bacillus pumilus* isolates

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Abstract

Spinetoram is the management of insect pests that affect vegetable crops growing in greenhouses and outdoors. It was produced first in 2007 by fermentation product of Saccharopolyspora spinose ($C_{42}H_{69}NO_{10}$). The current study describes the effectiveness of bacterial isolated from Iraqi environments, to remove spinetoram. Genetic analysis (16S rRNA) revealed that these strains are *Bacillus licheniformis* and *Bacillus pumilus*. Four temperatures (25, 30, 35, 40) ºC and pH values (5,7,9) were chosen for this study, and three concentrations of spinetoram (400, 800, 1200) mg/L were used. The spinetoram showed that the best biodegradation by consortium of Bacillus *licheniformis* and Bacillus *pumilus* was at a temperature of 35 ºC and pH 9 while the removal percentages were 99%, at 400 mg/L of spinetoram. The study's findings demonstrated the potential and importance of using aerobic biodegradation to remove spinetoram pesticides from agricultural soils. This consortium's capacity to break down pesticides makes it a valuable bioremediation tool.

Keywords: Spinetoram, pesticides, consortium of Bacillus *licheniformis* and *Bacillus pumilus*.

1. Introduction

The word "pesticide" describes any substance or combination of substances used to eradicate weeds and plants of dangerous pests, such as nematodes, insects, vertebrates, and other arthropods that pose a threat to our food supply, health, or comfort. The word "pesticide" refers to chemicals that alter the biological processes of living things that are considered pests, such as mold, fungi, insects, weeds, or noxious plants.

Pesticides are used extensively in most crop production areas to protect crops from possible yield losses and a drop in product quality by preventing pest infestations [1]. In last years, with the development of agriculture, pesticides have been hugely used

in different fields to obtain higher crop yields. Due to utilization rates and high residue levels, many pesticides remain in water, soil, air and crops causing environmental pollution [2,3]. Moreover, pesticides are highly toxic and able to cause chronic abnormalities in organisms including humans [4]. Spinetoram is an active component belonging to the spinosyn family of insecticides. Spinosyns are nicotinic acetylcholine receptor agonists (allosteric). It works by making contact or by ingesting the pesticide, and it is especially efficient against pest lepidopterans [5].

According to a study on the number of pesticides still present in the soil, pesticides contaminate the water, and agricultural chemicals like fertilizers and pesticides are carried by drain water that eventually empties into rivers and streams and then into the ocean. The increased levels of these pollutants have an impact on fish and other marine life [6]. When water is used for irrigation, pesticides are pushed deep into the ground (groundwater) and may even make their way into rivers. Enzymatic transformation is the main detoxifying technique and is primarily the result of biotic activities that are mediated by microorganisms and plants [7].

The biodegradation of pesticides is an innovative technique to lower pesticide contamination in an eco-friendly way for sustained environmental benefits. Microorganisms are known for their numerous uses and impact on human safety. Recent studies have demonstrated that isolated microorganisms from soil or water have the capacity to degrade pesticides [8]. Microbes can co-metabolize pesticides via microbial metabolic processes or utilize them as substrates for energy and nutrition during biodegradation [9]. Recent studies have demonstrated that bacterial consortia isolated from polluted environments can degrade organic pollutants [10,11,12,13].

Halophilic microorganisms are those that do well in conditions with high salinity. The ideal growth conditions for no halophilic microorganisms are below 2%, whereas halophilic and halotolerant bacteria can flourish in up to 30% NaCl. The aerobic, rod-shaped, gram-stain-positive, endospore-forming *Bacillus licheniformis* and *Bacillus pumilus* strains were isolated from drains and can adapt to harsh environments like high salinity. Halophiles also have a wide range of metabolic functions, including oxygenic and anoxygenic phototrophs, fermenters, aerobic heterotrophs, methanogens, sulfate reducers, and denitrifies [14].

Because of their capacity to degrade organic pollutants, halophiles have excellent potential for use in biotechnological

processes, particularly in bioremediation processes [15]. Environment: cillus contains the most common species in the environment; they are rod-shaped, aerobic, gram-stain-positive, and endospore-forming. Species of this genius easily adapt to a range of environments because of their ability to produce spores and survive in a variety of different environmental conditions [16]. This study's objective was to isolate and assess the efficiency of an aerobic bacterial strains capable of breaking down spinetoram.

2. Material and methods

2.1. Media and Chemicals

Spinetoram was bought from Dow AgroSciences (USA), and it was dissolved in acetone to produce a 2000 mg/L stock. Filter-sterilized and refrigerated, the stock solution was ready for use. The bacterial strains that degraded spinetoram were isolated and cultivated using nutrient broth, nutrient agar, halophilic agar M590, and halophilic medium, as stated by [17, 18].

2.2. Isolation of halophilic bacteria that degrade Spinetoram

Samples were taken from four drainages contaminated with Spinetoram (in December 2023), which have been belonging to agricultural areas in the Al-Kut city was in the Wasit Governorate / Iraq. Halophilic Agar M590 is the halophilic medium that was utilized in this investigation to isolate and cultivate halophilic bacteria. The same components of the halophilic media without agar were used to make the halophilic medium used as broth in the experiment [17, 18].

2.3. Identification of the halophilic bacteria that degrade Spinetoram

In addition to studying the morphological characters of the colonies cultivated on halophilic medium. PCR technology was used to diagnose isolated bacteria [19]. Forward primer 5'-AGAGTTTGATCCTGGCTCAG- 3' and reverse primer 5'-GGTTACCTTGTTACGACTT- 3’ were used for amplification. MEGA version 4 was used to perform molecular evolutionary analyses of the spinetoram-degrading bacteria based on 16S r-RNA genes [20].

2.4. Laboratory experiment

Sets of Erlenmeyer flasks of 250 ml holding 200 ml of the medium were prepared, the pH was adjusted to 7, and autoclaved at 121ºC and 1.5 bar for 15 min. Then sets of spinetoram with three

concentrations (400, 800, 1200 mg/L) were added to flasks, and then inoculum of a consortium of *Bacillus licheniformis* and *Bacillus pumilus* that were isolated and incubated after being activated in nutrient broth for five days.

For 21 days, the flasks were incubated at 150 rpm in a shaker incubator, and the growth was measured by recorded values of optical density by spectrophotometer at 600 nm every two days [21]. The experiment was repeated for pH 5, 7, and 9 at four different temperature degrees (25, 30, 35, and 40) ℃ for 21 days; optical density was measured for each one, and then 5 mL of the mixture was taken to test the biodegradation efficacy by HPLC analysis.

2.5. The HPLC

Concentrations used in the current study were 400, 800, and 1200 mg/L, which were measured according to Equation (1):

$$C1 \times V1 = C2 \times V2$$

Spinetoram content was determined by HPLC, and the percentage of spinetoram biodegradation was computed using the following equations (2,3) [22].

Percentage ratio of biodegradation = (Peak area of standard-Peak area of sample)/(Peak area of standard) × 100

The spinetoram residues concentration in samples were measured according to [22] from following equation:

Spinetoram concentration (ppm)= (Conc. of standard × peak area of sample)/(peak area of standard)

3. Results and discussion

3.1. Identification of bacteria

The PCR technique (16S rRNA) was used to identify the bacillus bacteria that were isolated from the drains. The results indicated that the bacteria were *Bacillus licheniformis* and *Bacillus pumilus* based on the nucleotide sequence of the 16S rRNA gene.

3.2. Impact of Temperature and pH on growth of consortium

Temperature and pH are two important environmental factors for pesticide degradation by microorganism. To increase microbial activity, increase the availability of pesticides to microorganisms, and thus achieve successful bioremediation of pesticide-contaminated environments, it is desirable to optimize the temperature and pH conditions [23]. The current study examined the effects of temperature and pH on spinetoram degradation. The results presented in Table 1 demonstrate that notable differences exist in the growth of consortium

at various temperatures and pH levels. The best and most significant means of consortium growth under 25 ºC, 30 ºC, 35 ºCand 40 ºC recorded at pH 9 were 0.180, 0.371, 0.420 and 0.119 nm, respectively. Also, the better and more significant means of consortium growth under pH (5, 7, 9) measured at 35 ºC were 0.335, 0.381, and 0.420 nm, respectively. The mean value of consortium growth was highest at 35 ºC and pH 9, which was 0.420 nm, and lowest at 40 ºC and pH 5, which was 0.082 nm (Figures 1 and 2).

Table 1: Mean value of consortium growth at various temperatures and pH degrees after incubation for 21 days, and LSD value with 400 mg/l of spinetoram.

Temp.	pH 5	pH 7	pH 9	LSD
25 °C	0.100 ± 0.021	0.130 ± 0.021	0.180 ± 0.026	0.127*
30 °C	0.203 ± 0.024	0.310 ± 0.125	0.371 ± 0.153	0.132*
35 °C	0.335 ± 0.014	0.381 ± 0.086	0.420 ± 0.121	0.123*
40 °C	0.082 ± 0.020	0.099 ± 0.027	0.119 ± 0.113	0.052 NS
LSD	0.082 *	0.127*	0.117*	----

* (P≤0.05), NS: Non-Significant.

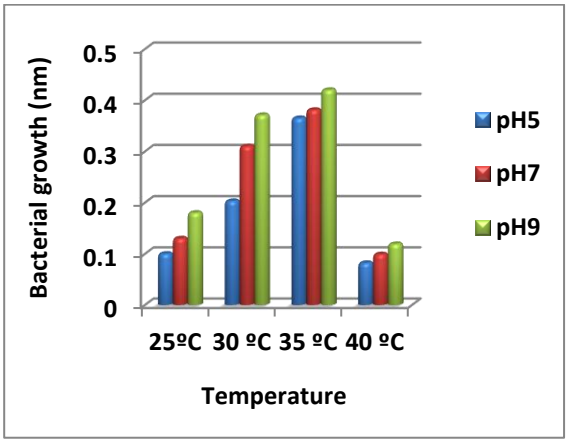


Figure 1: Mean value of consortium growth at various temperatures and pH values after incubation for 21 days.

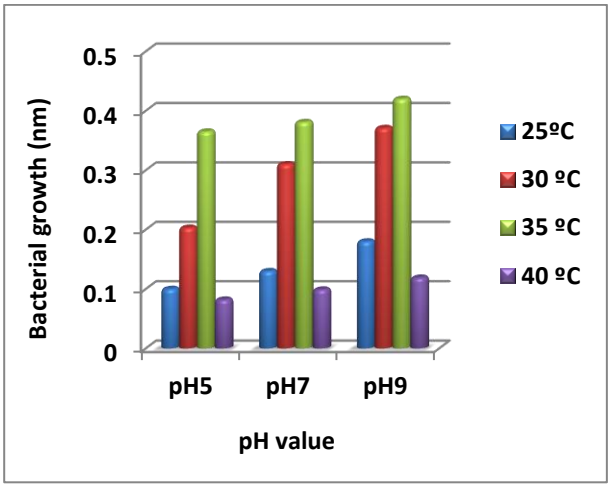


Figure 2: Mean value of consortium growth at various pH values and temperatures after incubation for 21 days.

Accordingly, the best degradation occurs at a temperature of 35°C for isolated bacteria. Similar studies showed that 35°C was necessary for the best degradation of spinetoram by *Bacillus licheniformis* and *Bacillus pumilus* [10, 24, 25].

These results indicate that low

temperatures decrease the degradability rate, and the higher temperature increases the degradability of spinetoram, but when the temperature rises above the threshold point of 37°C, it may increase the toxicity of microbial membranes. According to the data, 35°C would be the ideal temperature for better spinetoram degradation, as confirmed by HPLC.

In general, temperature is a dominant factor affecting the degradation of spinetoram; this is mainly because the growth of bacteria will be significantly affected when the temperature is high or low; bacteria spend energy on resistance in extreme conditions and spend it on building in normal conditions [26].

The pH of the environment where microorganisms are present has a major role in regulating their growth, and since pH influences the availability of nutrients, achieving the optimal pH is essential for the bioremediation of pesticide-contaminated ecosystems [27, 28]. The results indicated that the ideal pH for consortium growth in halophilic medium containing spinetoram is 9.

This conclusion is corroborated by the findings of [29], who found that when they used *Bacillus pumilus* to degrade pesticides in acidic and alkaline media, some essential enzymes that break down pesticides function best at high pH.

The pH levels have a direct impact on cell growth and metabolism, which in turn affects the capacity for degradation; alkaline pH promotes higher microbial biomass and enzymatic expression, which in turn aids the microbial community in adapting and developing gene-enzyme systems for the enhanced degradation of pesticides [24].

3.3. Impact of incubation time on biodegradation and growth

The results were shown in Table 2, a significant difference ($p<0.05$) between the consortium growth means for different incubation times. After twenty-one days of incubation, the selected isolated consortium demonstrated that the lowest growth was on the first day (0.230 nm), and the highest mean growth was on the thirteenth day (0.540 nm). From day one to day thirteen, the growth rate of the isolated consortium that was selected increased gradually and then started to decline (Figure 3).

Table 2: Average values ± standard deviation of consortium growth at 600nm at various incubation times and LSD value.

Incubation period Days	Mean± SD of bacterial growth consortium
First Day	0.240 ± 0.016
Third Day	0.325 ± 0.010
Fifth Day	0.385 ± 0.012
Seventh Day	0.430 ± 0.022
Ninth Day	0.496 ± 0.015
Eleventh Day	0.545 ± 0.011
Thirteenth Day	0.565 ± 0.032
Fifteenth Day	0.470 ± 0.021
Seventeenth Day	0.400 ± 0.016
Nineteenth Day	0.360 ± 0.024
Twenty-one Day	0.305 ± 0.030
LSD ≤ 0.05	0.034

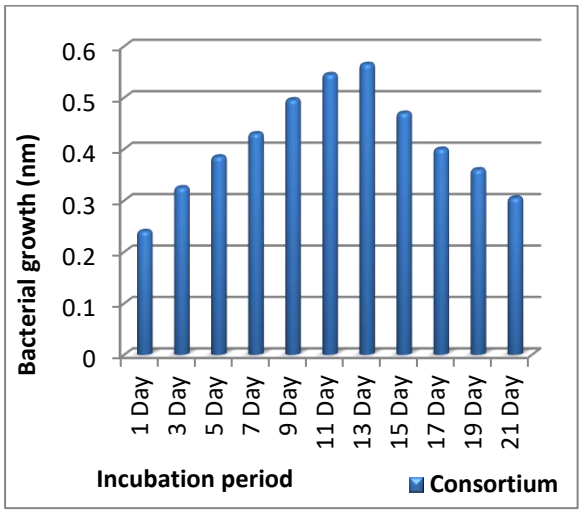


Figure 3: The average value of consortium growth over various incubation times.

The degradation of spinetoram significantly increased as the incubation times increased. It is commonly known that the increasing of the incubation period increases the number of viable organisms, particularly in media with minimal amounts of nutrients, and due to the production of intermediate compounds and metabolic products, as well as the reduction of contaminant concentration, which causes a lower pH in the media and then inhibits consortium growth [30].

3.4. HPLC analysis of biodegraded pesticide

To test the efficacy of degradation, a halophilic medium was used to inoculate the bacteria, with three different concentrations of pesticide (400, 800, and 1200 mg/L); the spinetoram was added to 200 mL of halophilic media at four different temperature degrees and three values of pH. The HPLC system was used to analyze these samples [31].

An analysis of a standard spinetoram solution was conducted; the standard solution's concentration was 100 mg/L, and its peak area was 951.90 per 2.69 minutes. Comparing figures of spinetoram were done according to the concentrations used in the study (Figure 4).

Table 3 displays the HPLC analysis results, explains the spinetoram concentrations, sample peak area, remaining concentration, and removal percentage for each sample.

Table 3: Peak area and removal percent of samples.

Temp.	Conc. of spinetoram (mg/L)	pH	Peak area (mAU.s)	% Removal	Remain conc. (mg/L)
25	400	9	1675.34	56	176
		7	2132.26	44	224
		5	3046.08	20	320
	800	9	7005.98	8	736
		7	7310.59	4	768
		5	7539.04	1	792
	1200	9	10851.66	5	1140
		7	1194.34	2	1176
		5	11308.57	1	1188
30	400	9	304.60	92	31.99
		7	609.22	84	64
		5	1142.28	70	120
	800	9	5711.4	25	600
		7	6549.05	14	688
		5	6777.52	11	712
	1200	9	9937.63	13	1044
		7	10280.52	10	1080.2
		5	10508.81	8	1104
35	400	9	38.08	99	4
		7	304.61	92	32
		5	494.99	87	52
	800	9	3039.03	60.09	319.26
		7	3921.83	48.5	412
		5	5231.64	31.3	549.6
	1200	9	8567.10	25	900
		7	9595.15	16	1008
		5	9937.83	13	1044
40	400	9	2246.48	41	236
		7	2513.01	34	264
		5	3198.38	16	336
	800	9	7234.44	5	760
		7	7424.28	2.5	780
		5	7539.04	1	792
	1200	9	10965.88	4	1151.9
		7	11307.99	1	1187.9
		5	11308.57	1	1188

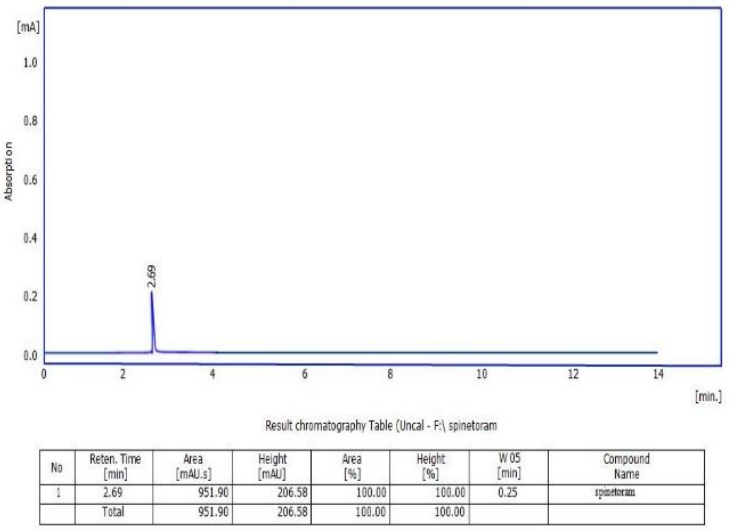
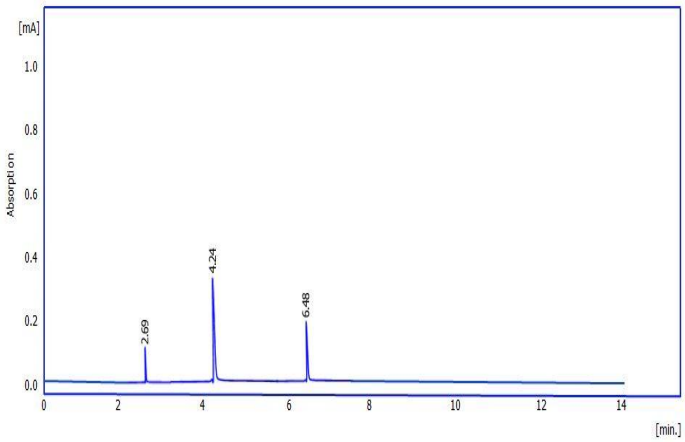


Figure 4: Peak area for 100 mg/L of spinetoram.

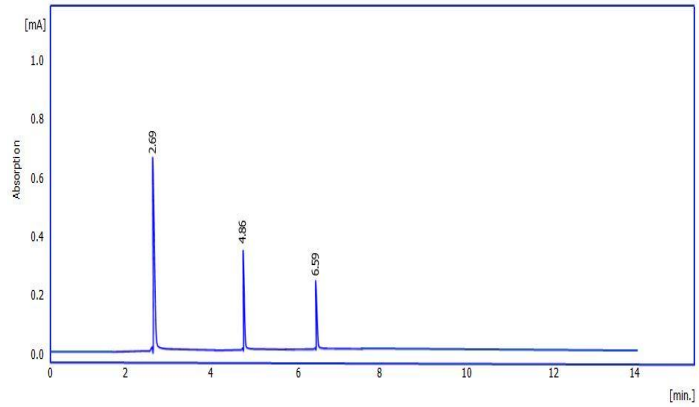
In this table, the dissociation of spinetoram in different concentrations with various temperatures and pH, where the best result was the breakdown of the spinetoram into its secondary components at a temperature of 35°C and pH 9 (Figure 5) with the concentration used 400 mg/L, while the lowest results were obtained at 40°C and pH 5 with 1200 mg/L, as demonstrate in (Figure 6). This indicates that the bacteria's ability to break down the spinetoram decreases with increasing spinetoram concentration and lower pH values.



Result chromatography Table (Uncal - F:\ B pH 9 (35) spinetoram 400 mg/L

No	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W 05 [min]	Compound Name
1	2.69	38.08	87.23	0.93	12.93	0.25	spinetoram
2	4.24	2854.29	379.86	69.63	56.32	0.25	
3	6.48	1206.81	207.43	29.44	30.75	0.25	
	Total	4099.18	674.52	100.00	100.00		

Figure 5: The highest removal of spinetoram.



Result chromatography Table (Uncal - F:\ B pH 5 (40) spinetoram 1200 mg/L

No	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W 05 [min]	Compound Name
1	2.69	11308.57	695.08	69.35	51.72	0.25	spinetoram
2	4.86	3265.09	384.75	20.02	28.63	0.25	
3	6.59	1732.28	264.12	10.63	19.65	0.25	
	Total	16305.94	1343.95	100.00	100.00		

Figure 6: The lowest removal of spinetoram.

The above table displays the biodegradation of spinetoram in different concentrations at various temperatures and pH. The best obtained removal percentage was 99% at temperature 35°C and pH 9 with 400 mg/L concentration (Figure 4). And the lowest removal percentage was 1% at 40 °C, pH 5 with 1200 mg/L (Figure 5). This indicates that the consortium is less effective at degrading the pesticide at lower pH values and higher spinetoram concentrations. The ideal environment for the biodegradation of pesticides varies depending on the compounds and organisms present, but it was found that the rate of degradation at acidic pH was slower than at alkaline and neutral pH due to the increased stability of different chemical groups at acidic pH [32].

In this study, the removal rates were very low with 1200 mg/L of spinetoram compared to the lower concentrations of 400 and 800 mg/L of spinetoram. This indicates that increasing concentration reduces the ability of used bacteria to break down spinetoram and thus gives fewer results even with optimum temperature 35°C and pH 9, where the removal rate was 25%. The rate of degradation was noticeably slowed down at higher concentrations, which seems to be connected to the pesticide's detrimental impact on bacterial growth [33].

The bioremediation efficacy of these isolated consortia was due to their metabolic enzyme system; certain enzyme systems that are tailored for the breakdown of structurally distinct pesticides may be

present in these organisms [34]. Genetic exchange between environmental strains may also contribute to the breakdown of pesticides by acquiring catabolic genes or particular enzymes [35]. Spinetoram can be harmful and toxic to microbes at certain concentrations. This is due to the depolarization of microorganisms' cell membranes brought on by spinetoram adsorption, which reduces the absorption of supplements and modifies the material released from cell metabolism. Eventually, the microorganisms' ability to survive will decline [26, 32].

The results agreed with [10,11,36]. which use *Bacillus* sp. to degrade pesticides and show 30–80% percentage of degradation to pesticides explained by HPLC analysis. The efficiency of the bacteria, the isolated strain type, and its environmental adaptation explain the small variation in the percentage of bio digestion [37].

5. Conclusion

The biodegradation of pesticides is an innovative technique to lower pesticide contamination in an eco-friendly way for sustained environmental benefits. The consortium of *Bacillus licheniformis* and *Bacillus pumilus* were isolated from an Iraqi agricultural drainage sample that was contaminated with spinetoram pesticide. The isolates were used in growth optimization studies, which included a variety of physicochemical parameters to encourage a higher rate of degradation and enable the application of the optimized parameters in the field. Using PCR technology, the isolates of bacteria were identified by their 16S rRNA gene nucleotide sequence. The consortium of halophilic bacteria has shown effective spinetoram pesticide removal rates. The optimal values of temperature and pH for the consortium of *Bacillus licheniformis* and *Bacillus pumilus* were found to be 35°C, and pH was found to be 9. The isolated bacteria obtained in this study is a valuable tool in the bioremediation of water polluted with spinetoram pesticide because it can utilize spinetoram pesticide as its carbon source.

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