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BIODEGRADATION OF HEXAMINE IN INDUSTRIAL WASTEWATER OF ANKLESHWAR UNIT IN GUJARAT STATE OF INDIA USING AN INDIGENOUS BACTERIAL ISOLATE PSEUDOMONAS KAN-X-012

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ABSTRACT: The industrial waste water generated during Hexamethylenetetramine (Hexamine) production mainly contains hexamine together with methanol and formaldehyde. Hexamine being bactericidal in nature and also resistant to biodegradation, it is pertinent to treat effluent having such compounds before discharging in natural streams. A bacterial strain capable of degrading effluents containing hexamine compounds from hexamine production unit of Ankleshwar was isolated. The isolate was put through phenetic and genetic identification on the basis of morphology, biochemistry, physiology and 16S rRNA analysis. It was a Gram negative rod capable of utilizing hexamine effluent as sole source of carbon and nitrogen. BLAST search of the 16S rRNA sequence of isolate (MTCC accession no 5833) exhibited maximum similarity (99.15%) with Pseudomonas taiwanensis having capability to degrade efficiently 50 % or more of 5000 ppm of hexamine in 22 days under aerobic conditions at alkaline pH. The isolate could reduce COD of the effluent by 70% and ammoniacal nitrogen by 50.0% when tested at 5L, 200 L and plant level bioreactors.

KEYWORDS: Biodegradation of Hexamethyltetramine – Hexamine - industrial waste water treatment - Pseudomonas sp. MTCC 5833

I. INTRODUCTION

Large amounts of pollutant containing effluents generated during production of hexamine derivatives pose severe, instant as well as long term ecological and environmental problems (Salleh et al. 2003; Liang 2009; Silbert 1999) dangerous for human health, animals and vegetation. Hexamethylenetetramine (hexamine) compounds are used as raw material in manufacture of phenolic resins and phenolic resin moulding compounds, finding applications mainly in prophylactic treatment of urinary tract infections, as solid fuel in catering industry and in food additive as a preservative(http://www.en.wikipedia.org/Hexamethylenetetramine/). Since the manufacture of hexamine involves large amounts of ammonia, formaldehyde, and methanol, these compounds along with hexamine form the major components of the effluents emerging from a hexamine manufacturing unit (Aladko et al. 2007; Alamadri et al. 2004; Kralj 2003).

The existing effluent treatment plant in Ankleshwar unit is aerobic activated sludge process. The effluent streams have very high chemical oxygen demand (COD), contributed mainly by hexamine together with formaldehyde and methanol which are antimicrobial by nature, and in addition has high ammoniacal nitrogen. Presence of these compounds in effluents renders the activated sludge treatment ineffective or makes it very slow (Kaszycki et al. 2001; Kaszycki and Koloczek 2002; Lofty and Rashed 2002; Lu and Hegemann 1998). The overall damage to ecosystem, flora and fauna caused by xenobiotic compounds has motivated researchers to develop new strategies for their removal from the contaminated environment (Porwal and Mane 2015). The application of microbial technology for the biodegradation of xenobiotics from biosphere has received much attention (Agrawal and Sahi 2015). Hexamine has been reported to be degraded / removed from effluents mainly by chemical methods (Bangxiao and Zhijun 1986; Chou et al. 1999; Golovashin et al. 2001). Biological methods reported are either ineffective (Kaszycki and Koloczek 2002; Saadatjou et al. 2010) or very slow (Hutnan et al. 2005; Gomolka and Gomolka 1984; Middelhoven and Doesburg 2007; Choulas and Adams 1972). Attempt has therefore been made in this study to develop a process of efficient microbial degradation of hexamine and associated chemical pollutants in the effluent discharge at the hexamine manufacturing unit at Ankleshwar through a bacterial isolate obtained from the hexamine contaminated soils.

II. MATERIALS AND METHODS

Screening of Hexamine degrading Microbes

Soil samples from packing area in the hexamine production unit , and sludge samples from common effluent treatment plant were collected. The samples were dispensed in sterile distilled water and streaked on nutrient agar plates containing sterilized hexamine effluent enough to make 1000 ppm concentration of hexamine in agar. Plates were incubated at 370 C for seven days. Colonies appeared on 3rd day were further grown on nutrient agar plates with increased concentration of hexamine up till 3000 ppm .Only one out of three isolates could grow at 3000 ppm hexamine concentration. This was selected for further study and

assigned KAN-X-012.

Morphological, biochemical and molecular Characterization of KAN-X-012

Characterization of the selected microbe was carried out on the basis of techniques described by Cappuccino (1996), including Gram's staining. The isolate was grown on Cetrimide agar having 0.5 % Cetrimide for selective and presumptive identification of Pseudomonas sp. (Lilly and Lowbury 1972). Slants of pure cultures of KAN-X-012 isolate was sent to Microbial Type Culture Collection and Gene bank (MTCC), Chandigarh for identification by sequencing of 16S rRNA and use of Identify Analysis .EzTaxon server 2.1 Software and Genebank database.

Optimization of incubation parameters for KAN-X-012

The isolated KAN-X-012 was adapted for growth on hexamine by gradually increasing its concentration up to 3000 ppm, in nutrient agar, Optimum growth was observed under aerobic condition at 370C and pH 8.0 to 9.0 indicating alkalophilic / alkali tolerant nature.

Effluent analysis before and after KAN-X-012 augmentation

Hexamine, formaldehyde and methanol contents present in the wastewaters and synthetic wastewater were analyzed as per method prepared at the 17th Joint (FAO/WHO) expert committee in Food Additives (JECFA 1973). Hexamine, formaldehyde and methanol concentration were also analyzed by using High Performance liquid chromatography (HPLC) with a Waters Bondapak C18 10 m 125 A Column (3.9 X 300mm); the mobile phase consisted of 100 % deionized water using refractometer as the detector Biodegradation process indicators including COD/BOD, ammoniacal nitrogen, TSS, TDS of original, inlet and outlet wastewater were analyzed by methods described by APHA (1992).

Biodegradation studies

Biodegradation studies were carried out in two phases. In first phase 2 L bioreactor with synthetic wastewater containing 6000 ppm of Hexamine concentration in 1 L deionized water was used, 100 ml of laboratory grown (0.125 OD) KAN012 was added to it, aeration was done continuously using fish pond aerators, pH was self maintained around 8.0 to 9.0 throughout for a period of 28 days at 370C. Samples were withdrawn daily and analyzed for COD, ammoniacal nitrogen, hexamine, formaldehyde, methanol concentration, MLSS and pH. A control was kept in the same way but without innoculum .The second phase of the study included the investigation to degrade effluent emanating from Ankleshwar unit at lab and plant scale using KAN012.

Scale-up of Biodegradation process at 5 L, 200 L level and plant level

Two plastic rectangular tanks of 5 Liter capacity each having feeding inlet and overflow outlet systems (Fig.1 a) was utilized as bioreactor for biodegradation using the simulated effluent. Aeration was done continuously using fish pond aerators. Nutrient di ammonium phosphate (DAP) was added initially to 1 L effluent to obtain 1 ppm concentration and inoculated with 100 ml laboratory grown (0.125 OD) KAN012. 500 ml of 2000 ppm hexamine containing effluent was added daily through a funnel initially, hexamine concentration was raised to 3000 ppm after 30 days and to 4000 ppm after 60 days. The overflow from the 2nd tank was collected at 22 days retention time. The pH was allowed to remain undisturbed by initially maintaining the pH around 8.0 to 9.0 using 1.0N Sulfuric acid throughout the entire cycle carried out in a room at 370C. Daily inlet and outflow samples were analyzed for COD, ammoniacal nitrogen and pH, and were plated on nutrient agar plates containing 4000 ppm hexamine for three successive months. The pilot bioreactor of 200 L capacity (Fig.1 b) was a two stage reactor made up of plastic tubs aerated continuously with help of cables connected to air compressor where, HRT was set up in such a way that overflow started at 22 days retention time. The pH remained stable around 8.0 to 9.0 at 370C R.T. Biodegradation was monitored by analysis of the inlet and outlet wastewater characteristics (COD, ammoniacal nitrogen, and pH). The process was scaled up at plant level (Fig.1 c) as to feed 50 M3 of effluent water daily in a four stage reactor system aerated by blowers. The overflow from one tank was sent to second tank and so on without any additions. Total HRT was 1200 M3 so as to get overflow after 22 days of retention. The pH remained self stable around 8.0 to 9.0 at R.T throughout.

Fig. 1a: Biodegradation process set up 5 L. | Fig. 1b: Biodegradation process scale up 200 L. | Fig. 1c: Biodegradation process scaled up at plant level.

III. RESULTS

Isolation of hexamine degrading microbes from soil samples

Screening of soil collected from packing area in the hexamine production unit Ankleshwar yielded three bacterial strains KAN-X -011, KAN-X-012 and KAN-X-013 growing on Nutrient Agar (N.A.) plates containing 1000 ppm hexamine, out of which only one: KAN-X-012 isolate was able to grow on 3000 ppm hexamine containing N.A. plates and Nutrient Broth flasks (Fig. 2a, 2b and 2c).

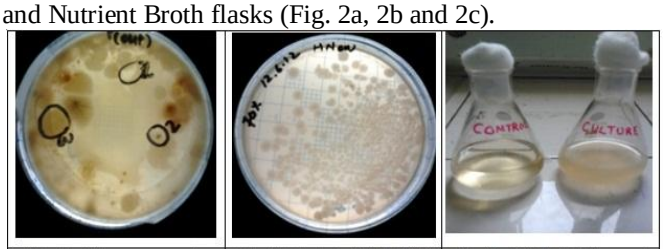

Fig. 2a:Primary screening on 1000 ppm Hexamine containing NAplate showing growth of three isolates KAN-X-011, KAN-X-012,KAN-X-013 . | Fig. 2b: Isolate KAN-X-012 growing on NA plate with 3000 ppm Hexamine. | Fig. 2c: KAN-X-012 growth in Nutrient Broth with 3000 ppm Hexamine.Control flask without isolate showed no growth on 3000 ppm Hexamine.

Morphological characteristics of isolate KAN-X-012

The morphological characterization of KAN-X-012, showed it to be Gram negative short rod that efficiently grew on Cetrimide agar as mucoid brownish red colonies which exhibited a brownish red fluorescence under ultra violet light suggesting it to be most likely a species of Pseudomonas family (Fig. 3 a-c).

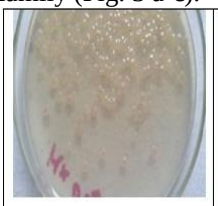

Fig. 3a: KAN-X-102 on Nutrient Agar showing brownish red pigment

Fig. 3b: KAN-X-012 on Cetrimide Agar

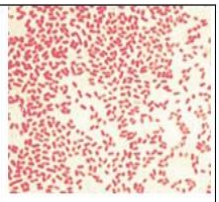

Fig. 3c: Microscopic view of KAN-X-012 : Gram Negative short rods

The gram negative property of KAN-X-012 was further confirmed by growth on MacConkeys agar and Eosin Methylene Blue (EMB) agar and also a positive result in 3 % KOH (sting) test (data not shown). It was motile non spore former and could grow at 42oC on various biochemical and physiological media as shown in Table 1 This confirms the isolate to be Pseudomonas sp.

Table 1: Taxonomic characteristics of the Hexamine degrading KAN-X-012 isolate.

Criteria	Observation
Morphological	Thin rods occur in chains, motile, pale pinkish colonies on nutrient agar
Gram staining	Gram negative
Physiological	
Gelatin liquefaction	Positive
Nitrate respiration	Positive
Oxidase	Positive
Catalase	Positive
Reduction of Nitrate	Reduced to Nitrite
Indole	Negative
H2S production	Negative
Starch hydrolysis	Negative
Casein hydrolysis	Positive
Urease	Positive
MR-VP test	Negative
NaCl tolerance	Growth up till 3 % NaCl
Citrate Utilization	Positive
Growth temperature	Growth at 42oC but not at 4oC
Production of acid	Produced acid but no gas from sucrose and xylose
3 % KOH sting test	Positive

Molecular identification based on 16 s rRNA analysis and phylogenetic relationship of KAN- X-012

Identification of KAN-X-012 by 16S rRNA sequencing as carried out at MTCC, Chandigarh India is presented in Table 2. It was identified using Identify Analysis .EzTaxon server 2.1 Software, to be a new Pseudomonas sp. (MTCC accession no 5833) exhibiting maximum pair wise similarity (99.15%) as well as closeness in the Blast Scores to Pseudomonas taiwanensis and greater than 98% similarity with 10 species of Pseudomonas. The species is being assigned a new specific name Pseudomonas kanoriaensis, based on the location of the isolation of this organism.

Table 2: Identification and Phylogenetic relationship of KAN-X-012 by 16S rRNA analysis and Identify Analysis .EzTaxon server 2.1 Software carried out by MTCC, Chandigarh

Rank	Species	Strain No. & Accession No.	Pairwise Similarity	Diff/Total Nt	MegaBLAST Score	BLASTN Score
1	*Pseudomonas taiwanensis*	BCRC 17751(T) EU103629	99.156	12/1422	2718	2718
2	*Pseudomonas monteilii*	CIP 104883(T) AF064458	99.088	13/1426	2718	2718
3	*Pseudomonas plecoglossicida*	FPC951(T) AB009457	99.018	14/1426	2710	2710
4	*Pseudomonas mosselii*	CIP105259(T) AF072688	98.948	15/1426	2702	2702
5	*Pseudomonas benzenivorans*	DSM8628(T) FM208263	98.317	24/1426	2630	2631
6	*Pseudomonas parafulva*	AJ2129(T) AB060132	98.247	25/1426	2623	2623
7	*Pseudomonas cremoricolorata*	NRC 0181(T) AB060136	98.247	25/1426	2623	2623
8	*Pseudomonas putida*	DSM 291(T) Z76667	98.175	26/1426	2611	2611
9	*Pseudomonas stutzeri*	ATCC 17588(T) CP002881	98.107	27/1426	2607	2607
10	*Pseudomonas oryzihabitans*	IAM 1568(T) D84004	98.107	27/1427	2607	2607
11	*Pseudomonas Japonica*	IAM 15071(T) AB126621	97.966	29/1426	2591	2591

Hexamine utilization by isolate KAN-X-012 Pseudomonas sp. (MTCC accession no 5833)

The results in Fig. 4 show ability of the isolate to utilize hexamine as sole source of carbon and nitrogen. It was grown on synthetic media containing 6000ppm hexamine, about 50 % reduction in hexamine and COD concentration was observed after about 28 days of incubation. Formaldehyde and methanol were not detected indicating that they were not liberated during biodegradation of pure hexamine. Control experiment without the isolate failed to show any reduction in hexamine concentration with no increase in MLSS values (data not shown). The results are indicative of ability of the isolate to utilize hexamine as a sole source of carbon and nitrogen.

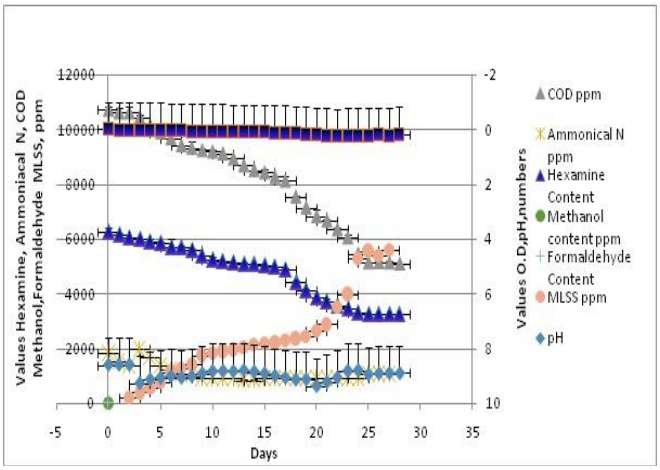

Fig. 4: Utilization of pure hexamine by the isolate KAN-X-012 Pseudomonas sp. (MTCC accession no 5833)

Data shown as mean + SEM of triplicate and are representative of two independent experiments SD >1 a Values of MLSS plotted are actual values multiplied by 101. As the isolate KAN-X-012 has typical morphological/rheological characteristic to remain in suspension the MLSS values observed were less but cell count on selective media and NA observed were high.

b Formaldehyde and methanol were not detected.

Further studies were carried out to examine ability of the isolate to reduce COD of the waste water. The analysis of wastewater used in the present study is shown in Table 1 where hexamine, formaldehyde and methanol contribute mainly to the COD of the effluent. The work was carried out first at 5L bioreactor level followed by its scale up to 200L level and subsequently at the plant level.

Table 3: Physicochemical analysis of incoming effluents from Ankleshwar unit.

Waste water constituents	Range
pH	9.5 to 10.2
Hexamine ppm	6000 to 10000
Formaldehyde ppm	300 to 500
Methanol ppm	7000 to 12000
C.O.D ppm	15000 to 20000
Ammoniacal N	1200 to 1500

Process performance: Biodegradation process at 5 L, 200 L reactor and at plant level

Fig. 5 shows the ability of isolate to bio remediate the polluted hexamine wastewater effluent down to lower limits at 5 L reactor. The untreated effluent was colorless and had a typical odor of ammonia but the outlet effluent was odorless and turbid due to bacterial growth. An average COD reduction of 53.27% (3684 ppm from wastewater containing 8196 ppm) with 46.15% reduction in ammoniacal nitrogen (317 ppm from wastewater containing 1120 ppm), and about 90% reduction in formaldehyde and methanol was observed with the pH of the effluent maintained at around 8.0 to 9.0.

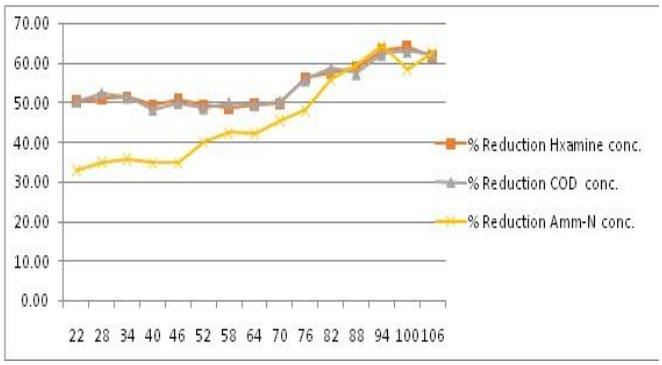

Fig 5: Percentage reduction in hexamine, COD and ammoniacal nitrogen at lab scale bioreactor set up with *KAN-X-012 Pseudomonas sp. (MTCC accession no 5833).*

The results obtained in bioremediation trials at pilot scale 200 L reactor are shown in Fig. 6 The untreated effluent was colorless and had a typical odor of ammonia but the outlet effluent was odorless but turbid due to bacterial growth. An average COD reduction of 74.35% (17450 ppm removed from wastewater containing 23200 ppm) was observed with 45.67% (535.5 ppm removed from wastewater containing 994.5 ppm) reduction in ammoniacal nitrogen, and an average 50% reduction in hexamine concentration (6705 ppm in wastewater). 90 % each of formaldehyde(4000 ppm in wastewater) and methanol(15500 ppm in wastewater) were found to be degraded, the pH of the tank was stable in range around 8.0 to 9.0 and MLSS values increased from 65 ppm to 1850 ppm and remained constant thereafter.

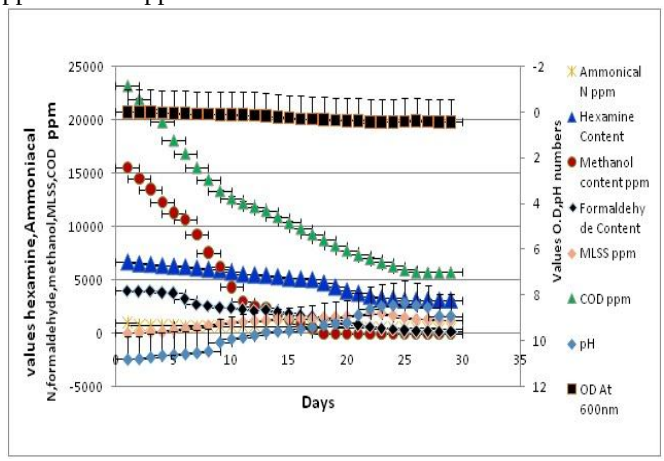

Fig 6: Reduction in hexamine, COD and ammoniacal nitrogen levels at 200 L pilot scale bioremediation set up augmented with KAN-X-012 Pseudomonas sp. (MTCC accession no 5833) .

*Data shown as mean + SEM of triplicate and are representative of two independent experiments SD >1

Fig.7 shows the results of hexamine wastewater treatment scaled up to plant level using the isolate. 50 M3 per day wastewater generated from hexamine plant was added in four stage aerobic tank system, the inlet and outlet physiochemical parameters of the wastewater were analyzed to observe the variance in parameters, Augmentation of the effluent with KAN-X- 012 Pseudomonas sp. (MTCC accession no 5833) at plant level reactor could reduce the COD by 70% and ammoniacal nitrogen by 56.7% bringing

both values within lower limits. (5442 ppm COD from wastewater containing 18286 ppm and 577 ppm ammoniacal nitrogen from wastewater containing 1345 ppm ammoniacal nitrogen).

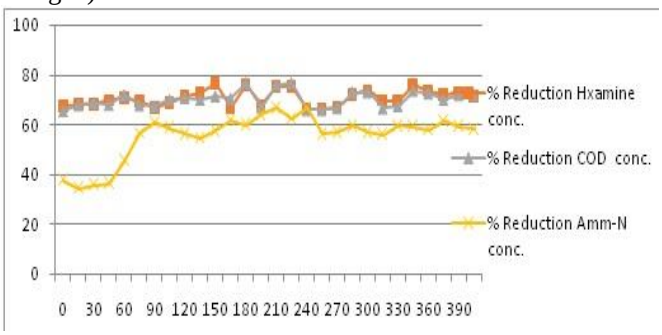

Fig 7: Percentage reduction in hexamine, COD and ammoniacal nitrogen at plant scale bioreactor set up with KAN-X-012 Pseudomonas sp. (MTCC accession no 5833) isolate.

IV. DISCUSSION

Hexamine is a slowly biodegradable compound, hydrolyzing in acid environment to ammonium and formaldehyde. Formaldehyde being toxic under normal conditions, treatment of hexamine containing effluent is of utmost importance. Scanty reports are available in literature regards biodegradation of hexamine. About 95% removal of hexamine reported using anaerobic baffled reactor (Hutnan et.al. 2005). Present study reports aerobic degradation of hexamine to certain level using a bacterial strain isolated from the soil samples collected from factory premises hexamine production plant Ankleshwar Gujarat and identified as Pseudomonas sp. The comparison of results with other reported methods indicate this process to be more efficient and effective .M. Hutnan et .al. (2005) reported about 85- 95% removal of hexamine (initial hexamine conc.1200 mg/l in influent) using ABR and UASB bioreactors. A novel Electro-Fenton method has been successfully used to remove approximately 66 mg/L (0.47 mM) in wastewater containing approximately 2500 mg/L (18.1 mM) hexamine by Chou et.al (1999).The reverse osmosis method reduces hexamine concentration to 0.2 % in wastewater containing 1 % hexamine (Bangxiao and Zhijun 1986). Hexamine has been reported to get hydrolyzed to formaldehyde and ammonia under acidic conditions and formaldehyde being biodegradable can be treated under aerobic conditions. (Gomolka and Gomolka 1984). Since pH of the effluent of Ankleshwar unit used in the present study remains at around 8-9, acidic hydrolysis of the hexamine to formaldehyde may not be the cause of reduction in COD. Besides the isolate when grown on pure hexamine showed about 50 % reduction in hexamine concentration (Initial hexamine conc. 6000 ppm in influent) (Fig. 4a) under aerobic condition supporting the observation that reduction in COD and hexamine observed in bioreactor study has been brought about by aerobic degradation of hexamine by the isolate.

This is to the best of our knowledge first report of the aerobic degradation of hexamine using a bacterial isolate.

V. CONCLUSION

A microbe isolated from soil samples collected from hexamine production plant Ankleshwar Gujarat was found to be efficient in hexamine wastewater biodegradation. The isolated microbe was identified as Pseudomonas sp. Gram – ve rod, closely associated with Pseudomonas taiwanensis. It has been deposited at MTCC Chandigarh (accession no 5833.) The isolate was efficient in degrading xenobiotic pollutant mainly hexamine as present in hexamine wastewater. COD values were reduced by 70% (approximately 14000 ppm removed from wastewater containing 20000 ppm COD) and ammoniacal nitrogen reduction was around 50.0 %.(approximately 750 ppm removed from wastewater containing 1500 ppm). The isolate can be explored for further studies in bioremediation of xenobiotic industrial pollutants.

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