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PRODUCTION OF MODERATELY HALOTOLERANT, SDS STABLE ALKALINE PROTEASE FROM *BACILLUS CEREUS* MTCC 6840 ISOLATED FROM LAKE NAINITAL, UTTARANCHAL STATE, INDIA

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ABSTRACT

A moderately cold active, extracellular alkaline protease producing bacterium was isolated from a fresh water lake. The isolate was found to be a gram-positive, rod shaped organism later identified as *Bacillus cereus* MTCC 6840. The bacterium produced the maximum amount of enzyme when allowed to grow for 24 h at temperature 25º and pH 9.0. Among a variety of substrates used, fructose as a carbon source and a combination of yeast extract and peptone as nitrogen source, supported the maximum protease production by the organism (120 U/ml). Fe^{++} and Co^{++} stimulated the enzyme activity whereas Ca^{++}, Cu^{++}, K^{+}, Mg^{++} and Mn^{++} inhibited it to different extents. The protease was found to be highly stable in the presence of NaCl, SDS and acetone. Treatment with EDTA and PMSF resulted in the considerable loss of enzyme activity. The enzyme was found to be optimally active at pH 9.0 and temperature 20ºC.

Key words: Alkaline Protease, *Bacillus cereus*, Enzyme activity

INTRODUCTION

Proteases represent one of the largest groups of industrial enzymes and account for 60% of the total worldwide sale of enzymes. The vast diversity of proteases, in contrast to the specificity of their action, has attracted the attention of scientists in an attempt to exploit their biotechnological prospects (25). The use of alkaline proteases in detergents, food, leather and silk industries has remarkably increased in recent years (12). Of all proteases, alkaline proteases produced by *Bacillus* species are of utmost importance due to the stability of their enzymes under different environmental conditions (14). While several proteases from alkalophiles have been studied comprehensively, proteases from halophiles and moderate halophiles have not been investigated in detail (12). Moderate halophiles constitute a very interesting group of organisms with great potential for use in biotechnology because of their high activities at high salt concentrations (31).

The high activity of psychrophilic enzymes at low and moderate temperatures offers potential economic benefits. Recently, there has been an increased emphasis on cold active alkaline proteases, which are active at ambient temperature so that many of the industrial processes utilizing them could be made more cost effective by reducing the consumption of energy (1). Microorganisms that can degrade proteins rapidly at low temperatures are necessary for treatment of protein containing wastewater because the temperature of such waste-water drainage is relatively low (approx. 5-25ºC). It is, therefore, important to study the novel cold active, halo tolerant proteases owing to their industrial utility. The present report on the production and partial characterization of a moderately cold-active, salt-tolerant alkaline protease secreted by *Bacillus cereus* MTCC 6840, isolated from lake Nainital, is a part of our attempt to identify protease-producing bacteria from the lakes of Uttaranchal, India.

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MATERIALS AND METHODS

Chemicals

All the chemicals used, unless stated otherwise, were of analytical grade and procured from Central drug house (CDH) chemicals Pvt. Ltd., India, Sisco Research Laboratory (SRL) Pvt. Ltd. India and Himedia Pvt. Ltd., India.

Isolation of bacterial strain

Water samples collected from five different lakes namely, Nainital, Bhimtal, Naukuchiatal, Sattal and Khurpatal of Uttaranchal state, India, were serially diluted and plated on milk agar plates. Based on the clear zone formation on these plates, bacterial strain GKSP1, that was isolated from lake Nainital and later identified as *Bacillus cereus* strain No. MTCC 6840 by Institute of Microbial Technology (IMTECH), Chandigarh, India, was selected for further study.

Growth and Optimization of Culture conditions

The test organism was grown in Erlenmeyer flasks with GYP medium (18) at pH 7.5 inoculated with 10% (v/v) of 24 hr old seed culture prepared in the same medium at 30ºC for 48 h in an orbital shaker at 165 rpm. Five ml of medium was withdrawn at a regular interval of 4 h and its absorbance was measured at 610 nm. The contents were then centrifuged at 10,000 rpm at 4ºC for 10 minutes and protease activity was checked in the supernatant.

Effect of different carbon sources on enzyme production by *Bacillus cereus* MTCC 6840 was studied by substituting glucose in the medium with different carbon sources (starch, sucrose, maltose, mannitol, glycerol, lactose, xylose, galactose and fructose). After determining the optimum carbon source, different nitrogenous compounds; casein, yeast extract, beef extract, peptone, gelatin, tryptone, $(NH_4)_2SO_4$, KNO_3 and $NaNO_3$ were tested for their ability to support the maximum production of enzyme. Culture conditions were further optimized by growing the isolate at different temperatures (from 20 to 45ºC) and pH (from 5 to 10) and determining the enzyme activity in the supernatant. All the experiments were performed in triplicate, and the standard error in all the experimental results was within 5%.

Protease assay

The protease activity was assayed by the standard method with some modifications using casein as the substrate (21). The reaction mixture, containing 4 ml of 2.5% (w/v) casein in 50 mM of Tris-HCl (pH 7.5) buffer and 1 ml of suitably diluted enzyme was incubated at 30ºC for 5 min. The reaction was terminated by the addition of 5 ml of 5% (w/v) trichloroacetic acid and the contents were filtered through Whatman filter paper. The absorbance (A) of filtrate was measured at 275 nm. One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 µg of tyrosine/ml/min under assay conditions.

Partial characterization of the enzyme

Protease activity was determined at different temperature (15 to 55ºC) and pH (from 5 to 10). The effect of different organic solvents (methanol, ethanol, chloroform, Isopropyl alcohol, n-butanol, acetone, formaldehyde and diethyl ether) on enzyme activity was checked by mixing these solvents at a concentration of 50% (v/v) to the enzyme solution and incubating the mixture for 2 h at 4ºC prior to enzyme assay. The effect of cations (Ca^{++}, Fe^{++}, Cu^{++}, Na^{+}, K^{+}, Mg^{++}, Mn^{++}, NH_4^{+} and Co^{++}), EDTA and various commercial detergents (Surf Excel and Rin Shakti, Hindustan Lever Ltd., India; Ariel and Tide, Procter and Gamble, India; and Fena, Fena P. Ltd., India), on enzyme activity was studied by adding them in the reaction mixtures at a concentration of 1 mM, 5 mM and 1% (w/v), respectively. In a similar manner, the effect of PMSF (5 mM) and SDS (1%, w/v) on enzyme activity was also studied. NaCl was added in the reaction mixture at different concentrations from 1 to 10% (w/v) and the enzyme activity was determined in each case.

Identification of bacterial isolate GKSP1

After performing the biochemical tests (Table 1) for bacterial characterization, the isolate was sent to Microbial type culture collection unit of Institute of Microbial Technology (IMTECH), Chandigarh, India for its complete identification.

RESULTS AND DISCUSSION

Our experimental results show that the maximum amount of enzyme (60.0 U ml^{-1}) was produced by the bacterium in its late log growth phase after 24 h of incubation, and a reduction in enzyme production was noted beyond this period (Fig. 1). There are supporting observations that protease production is characterized by an abrupt decrease of enzyme activity at the late exponential phase of growth culture. The decrease in protease activity is caused by the cessation of enzyme synthesis together with a rapid deactivation of the existing enzymes (4). In comparison to early reported halotolerant protease producing *Bacillus* sp. (12), the bacterium isolated in the present study secrets the enzyme at an early stage of growth. Fructose as a carbon source was preferred over glucose to support the maximum protease production in the present case. This indicates that the isolate differs significantly in its energy requirement for protease production in comparison to most of other bacilli, which utilize glucose as the optimum carbon source (8,13,19). In contrast to our observations, Kumar *et al*. (19) have reported the inhibitory effect of fructose over protease production. Starch has also been reported as optimum carbon source for *Bacillus* sp. (28), *Bacillus* sp. JB-99 (11) and *Bacillus cereus* BG1 (9) for protease production. Contrary to these findings, it was the least preferred choice of our organism for protease production. It may be due to its failure to channelize the energy requirement for protease production through hydrolysis of this complex

Table 1. Biochemical characterization of *Bacillus cereus* MTCC 6840.

Name of the test	Observation
*Gram's Stain	Positive
Endospore staining	Negative
*Cell shape	Rods
*Density	Opaque
*Elevation	Convex
*Margin	Irregular
Configuration	Lobate
Pigments	Not produced
H_2S Production	Negative
MacConkey Agar growth	Negative
Fluorescence	Negative
Motility	Positive
*Catalase	Positive
Oxidase	Positive
Methyl Red	Positive
Vogas Praskaur	Negative
Indole	Negative
Growth on Furazolidone agar	Negative
Citrate utilization	Negative
*Starch	Not hydrolysed
*Casein	Hydrolysed
*Gelatin	Hydrolysed
Nitrate reduction	Negative
Anaerobic growth	Negative
*Urease	Not Produced
Acid production from	
Glucose	Positive
Arabinose	Negative
Mannitol	Negative
Xylose	Negative
Meso-inositol	Negative
Raffinose	Negative
Rhamnose	Negative
Salicin	Positive
Sucrose	Negative
Galactose	Negative
Fructose	Positive

* These tests were carried out in our laboratory whereas rest of the analysis was performed at MTCC, IMTECH, Chandigarh, India.

carbohydrate. The test organism preferred organic nitrogen sources to inorganic nitrogen sources for protease production. A combination of Yeast extract and peptone was found to be the optimum nitrogen source for protease production in this case. A similar effect of the combination of these nitrogen sources on protease production by other *Bacillus* spp. have been reported by previous workers (7,19). The use of optimum carbon and nitrogen sources together thus enhanced the total protease production by *B. cereus* MTCC 6840 to 90 U/ml (Figs. 2 and 3).

Temperature has profound influence on production of protease by microorganisms. Although the mechanism of temperature control over enzyme production is not well understood (3), a link between enzyme synthesis and energy

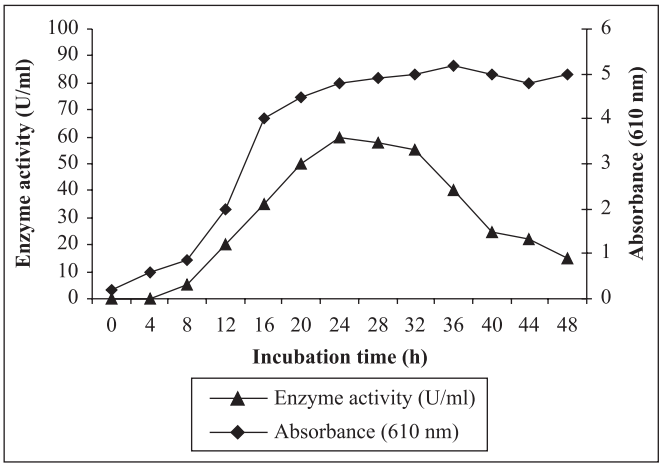

Figure 1. Protease production by *B. cereus* MTCC 6840 at different interval of growth. The isolate was grown in liquid medium for 48 h. The optical density of the culture broth and the protease activity in the supernatant were determined at regular interval of 4 h.

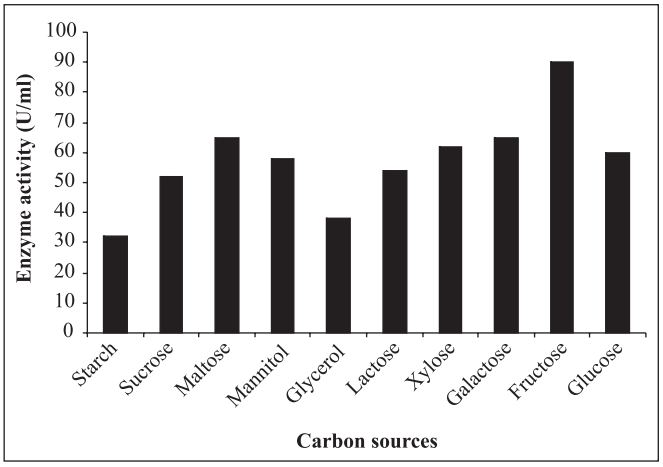

Figure 2. Effect of carbon sources on protease production. The isolate was grown in the presence of different carbon sources in the liquid medium. This was centrifuged after 24 h of growth and the enzyme activity was measured in the supernatant.

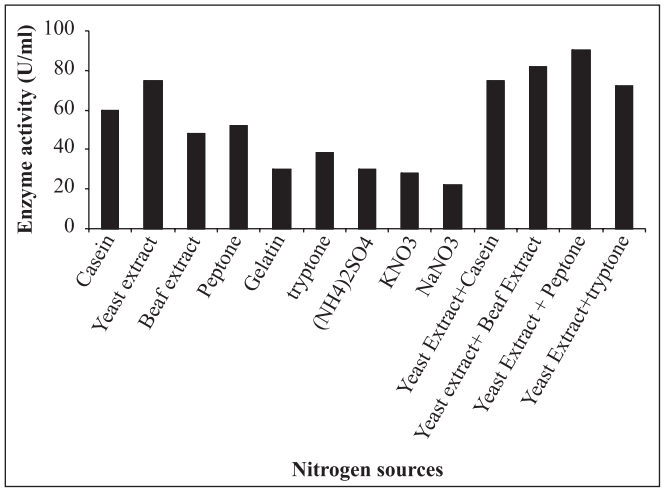

Figure 3. Effect of nitrogen sources on protease production. Different organic and inorganic nitrogenous compounds were included in the medium and the isolate was allowed to grow for 24 h. The extracellular enzyme production was recorded in each case.

metabolism has been reported in *Bacillus*, which is controlled by temperature and oxygen uptake (6). In our study, although the bacterium was able to grow well at temperature up to 45ºC, it preferred lower mesophilic range of temperature for enzyme synthesis with maximum production (98 U/ml) being recorded at 25ºC (Fig. 4). This is slightly higher than optimum temperature of alkaline protease producing psychrophilic *Bacillus cereus* sp. (26) and *Pedobacter cryoconitis* (22). The pH of culture medium strongly affects many enzymatic processes and transport of compounds across the cell membrane (19). Borriss (2) have reported the pH between 9.0 and 13.0 as optimum for alkaline proteases. The isolate *B. cereus* MTCC 6840 was able to grow well between pH 6.0 to 10.0 and produced the enzyme maximally at pH 9.0 (Fig. 5). The enzyme production could thus be increased to a level of 120 U/ml after growing the test organism under these optimized conditions of temperature and pH. These optimized environmental conditions indicate the alkalotolerant, moderately psychrohilic nature of the isolate *B. cereus* MTCC 6840.

The protease derived from *B. cereus* MTCC 6840 was found to be optimally working at 20ºC (Fig. 6) and only 18% loss in the activity was reported when the reaction was carried out at 15ºC.

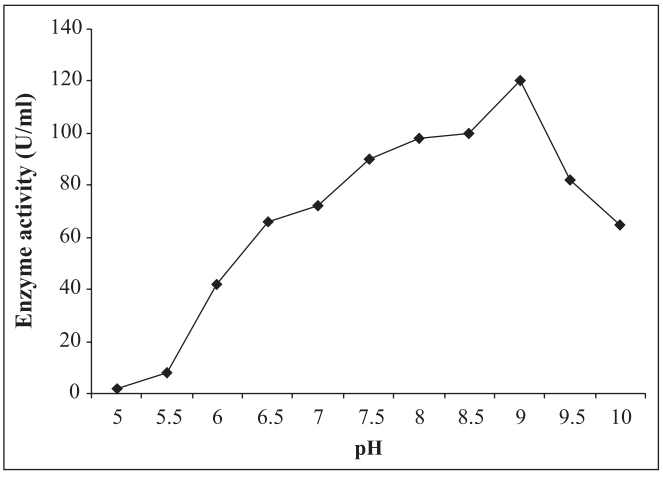

Figure 5. Effect of pH on protease production. The pH of the culture medium was varied from 5 to 10 and the corresponding effect on enzyme production was recorded.

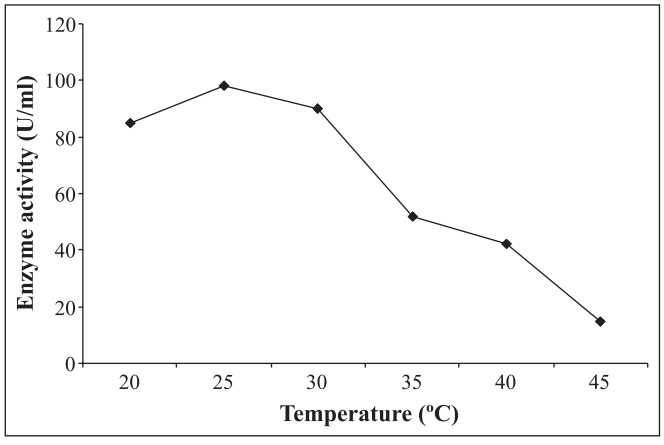

Figure 4. Effect of temperature on protease production. The isolate was grown at different temperatures (20 to 45ºC) and the enzyme production was determined in each case.

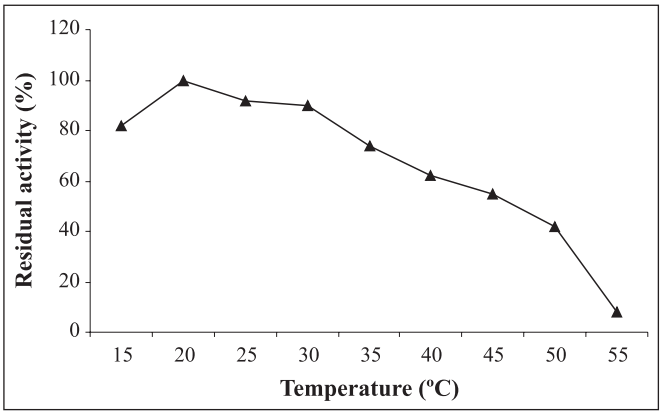

Figure 6. Effect of temperature on enzyme activity. The optimum temperature requirement of the enzyme was determined by carrying out the enzyme assay at different temperatures and calculating the residual activity (%) in each case.

Similarly the activity decreased slowly up to 30ºC but a rapid loss in the enzyme activity was noticed above this temperature. The findings thus indicate the moderately cold-active nature of the enzyme. There are reports of isolation of cold-active enzyme producing *Bacillus cereus* spp. (16,26) and *Pseudomonas* spp. (30) but, unlike the present case, their temperature optima are much higher than the protease of *B. cereus* MTCC 6840. However, much similar to our findings, *Bacillus* sp. MLB-2 (1) secreted cold active protease active in the temperature range of 20-35ºC. The optimum pH for the activity of the protease of the test organism was 9.0 (Fig. 7). A large number of alkaline proteases from *Bacillus* spp. are known to exhibit the maximum activity at pH 9.0 (5,17,19) and thus the enzyme produced by our isolate can also be considered as an alkaline protease.

All the organic solvents tested in the present study, negatively affected the enzyme activity to different extents and a remarkably high residual activity (92%) was reported in the presence of acetone. In the presence of methanol, ethanol, chloroform, isopropyl alcohol, n-butanol and diethylether the remaining activity was 52, 72, 66, 52, 48, and 42%, respectively, while it was only 22% with formaldehyde. There are few reports on the stability of proteases in organic solvents (23,24). The reasons for low activity of protease in organic solvents may be due to limited dispersion of enzymes, the partial denaturation of enzymes and the reduced flexibility of proteins in anhydrous solvents (18). Most of the enzymes are easily inactivated or denatured in organic solvents, and proteases, which are active in organic solvents, have potential application in catalyzing synthetic reactions (15,23). Although all the commercial detergents tested negatively affected the enzyme activity, the enzyme restored a significant 85% activity in the presence of Surf Excel brand of detergent. The residual activities in the presence of Fena, Rin shakti, Ariel and Tide brand of detergents were 65, 76, 71 and 50%, respectively. The results are significant in view of the facts that only few proteases from *Bacillus* sp. have been reported to be stable in the presence of detergents (10,27).

Of the various metal ions tested, Fe^{++} and Co^{++} stimulated the enzyme activity whereas other ions exerted a negative effect on it (Table 2). This enhancement in activity may be attributed to the ability of these ions to protect the enzyme against thermal denaturation and maintain its (enzyme) active conformation. Like the present case, Fe^{++} ions in the reaction mixture increased protease activity of *Bacillus coagulans* PB-77 (8) and *Bacillus* sp. APR-4 (19). EDTA and PMSF reduced the protease activity of *B. cereus* MTCC 6840 to 52 and 21%, respectively. Very few workers have reported such unusual inhibition of bacterial protease by both EDTA and PMSF (16,20). It can, therefore, be concluded that the protease studied in the present case, is a metal-activated enzyme with a loose interaction with metal, and which seems to have a serine residue at the active site. Addition of NaCl, up to a concentration of 5% (w/v), had no effect on enzyme function. However, the enzyme activity gradually decreased on further addition of salt and a 60% reduction in the activity was reported when NaCl concentration was increased to 10% (Table 3). The aforesaid observations suggest the moderately salt-tolerant nature of this enzyme. There are reports on salt-tolerant protease producing mesophilic or thermophilic *Bacillus* spp. (12,29) but the information on such proteases from cold active bacteria is still very limited. Besides temperature and pH stability, a good detergent protease should be able to work in the presence of surface-active agents. Following treatment with SDS, only 25% loss in the activity of protease of *B. cereus* MTCC 6840 strengthens its candidature to be used as a potential additive for commercial detergents.

The present work, therefore, is an attempt to unravel the untouched microbial diversity of water resources of Uttaranchal in terms of some functional attributes. The protease, produced by the test organism, owing to its alkaline nature and ability to

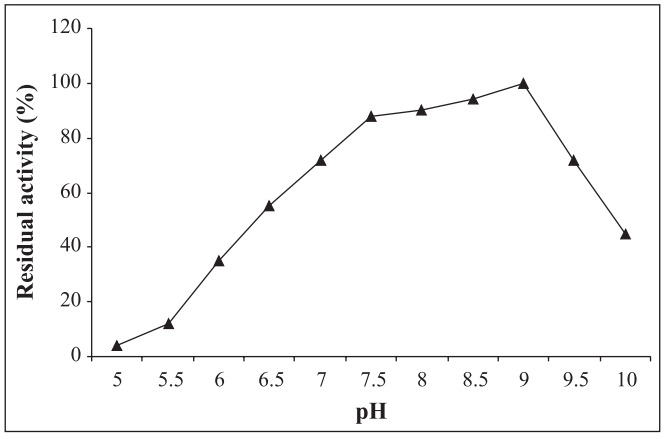

Figure 7. Effect of pH on enzyme activity. The pH of the reaction buffer was altered and protease assay was performed to determine the residual activity (%) in each case.

Table 2. Effect of cations on protease activity.

Cations	Residual protease activity (%)
Control	100
Ca^{++}	92
Cu^{++}	82
Fe^{++}	112
Na^{+}	100
K^{+}	98
Mg^{++}	75
Mn^{++}	58
$NH4^{+}$	88
Co^{++}	108

work in the presence of SDS, salt, solvents and various commercial brand detergents, seems to be of considerable use in industrial processes (such as detergent formulations, sewage treatment, leather processing) carried out at moderately low temperatures. However, further studies on its stability under various conditions are suggested before its industrial use.

Table 3. Effect of NaCl on protease activity.

NaCl (%)	Residual protease activity (%)
1	100
2	100
3	100
4	100
5	100
6	86
7	72
8	54
9	45
10	40

RESUMO

Produção de protease alcalina moderadamente halotolerante e resistente a SDS, produzida por *Bacillus cereus* MTCC6840 isolado do lago Nainital, Estado de Uttaranchal, India

Uma bactéria produtora de protease alcalina extracelular, moderadamente ativa no frio, foi isolada da água de um lago. Trata-se de um bacilo Gram positivo, identificado como *Bacillus cereus* MTCC6840. A maior produção da enzima foi em 24h a 25ºC e pH 9,0. A produção máxima de protease (120 U/ml) ocorreu quando foi utilizada frutose como fonte de carbono e uma combinação de extrato de levedura com peptona como fonte de nitrogênio. Fe^{++} e Co^{++} estimularam a atividade da enzima, enquanto Ca^{++}, Cu^{++}, K^{+}, Mg^{++} e Mn^{++} tiveram efeito inibitório, com intensidades diferentes. A protease permaneceu estável na presença de NaCl, SDS e acetona. O tratamento com EDTA e PMSF causou uma significativa perda na atividade. A enzima apresentou atividade ótima em pH 9,0 e temperatura de 20ºC.

Palavras-chave: Protease alcalina, *Bacillus cereus*, atividade enzimática

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