RESEARCH ARTICLE

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Isolation and screening of biopolyester (poly-β-hydroxybutyrate) producing bacteria from compost samples in Bangladesh

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ABSTRACT

Poly-β-hydroxybutyrate (PHB) is the simplest member of polyhydroxyalkanoates (PHAs) that are biological polyesters or biopolymers produced by a wide variety of bacteria as an intracellular storage material of carbon and energy. Compost is one of the richest sources of microorganisms; therefore, an attempt was made to isolate PHB producing bacteria from compost samples. Out of six compost samples, bioslurry showed the highest mesophilic bacterial count of 3.75×10^9 cfu/g. A total of 48 mesophilic bacteria were isolated by growing samples on nutrient agar medium at pH 7.0 and a temperature of 37°C. All isolates were purified and screened for PHB production using lipophilic stains such as Nile red, Sudan black B, Acridine orange. 16 out of 48 isolates exhibited PHB production ability after screening by plate assay. PHB granule formation was also confirmed by observation under a fluorescent microscope. The number of PHB producer and accumulators in different compost samples were in the order of Bioslurry > Cow dung > Cotton seed > Tobacco dust. Based on morphological, cultural and biochemical characteristics, all isolates were assigned to four genera viz. *Bacillus, Micrococcus, Veillonella and Pseudomonas*. Batch fermentation in shake flask was carried out using glucose as carbon source. By analyzing photomicrographs of 24-hour old cultures, 7 out of 16 bacterial isolates were found with a good PHB accumulation capacity. These bacteria are being currently studied for biopolymer production using different carbon sources and renewable biomass.

Key words: compost, poly-β-hydroxybutyrate, nile red, sudan black B, acridine orange, screening

Introduction

The exponential growth of the human population has led to the accumulation of huge amounts of non-degradable waste materials across our planet. Living conditions in the biosphere are therefore changing dramatically, in such a way that the presence of non-biodegradable residues is affecting the potential survival of many species (Luengo et al. 2003). One of such environmental threats is synthetic polymer or plastic. In order to find alternative materials, researchers have developed fully biodegradable plastics, such as polyhydroxyalkanoates (PHAs).

PHAs are a group of biodegradable polymers of biological origin. They are attractive substitutes for conventional petrochemical plastics as a result of having material properties similar to various thermoplastics and elastomers, as well as being completely biodegradable upon disposal under specific environmental conditions (Sudesh et al. 2000). PHAs are accumulated as a carbon and/or energy storage material in various microorganisms usually under the condition of limiting nutritional elements such as N, P, S, O, or Mg in the presence of excess carbon source. Many microorganisms have the ability to synthesize and degrade these macromolecules enzymatically (Mergaert et al. 1992). PHA is now being produced industrially by several companies over the world for use as natural, biodegradable, and biocompatible thermoplastics with a variety of potential applications (Kalia et al. 2000).

Active search and studies on an ideal organism for polyhydroxyalkanoate (PHA) production to replace petrochemical based plastics have been carried out over the last 30 years. A few studies reported on the isolation of PHA accumulating bacteria from the environments (Redzwan et al. 1997; Alias & Tan, 2005; Berlanga et al. 2006) utilizing oils

and glucose as carbon and energy source. However, there is a need to explore local, new bacteria for their capability in producing PHAs. The best PHB producing species should satisfy several demands such as the fast-growing population, being able to utilize cheap carbon and having a high production rate. There are two ways of acquiring new bacterial species to meet the above demands: to isolate from the natural environment and design genetic recombination strains.

While isolating PHB-accumulating bacteria from nature, it is necessary to screen rapidly a wide collection of bacteria in a short time. Stains specific to PHB are made use of in the detection of the granules. Viable colony staining technique has been suggested as a method for rapid screening of PHB accumulating bacteria. Lipophilic dyes such as Sudan black B, Nile blue A, and Nile red have been traditionally used for the first-line screening of PHB-containing bacteria (Kranz et al. 1997; Ostle & Holt, 1982; Schlegel et al. 1970). For this reason, attempts have been taken in this study to isolate PHB producing bacteria from a rich natural microbial source such as compost. The production of natural microbial polymers with biodegradable properties meets the need for environmental protection.

Materials and Methods

Sample collection

Six types of compost samples were collected from Multipurpose Attractive & Trusty Initiative (MATI), Bhawal Mirzapur, Gazipur, Dhaka, Bangladesh. The industry has two types of composting facilities-(i) aerobic, (ii) anaerobic. The raw materials used in that composting industry are mainly poultry slurry, cow dung, tobacco dust, cotton seed and water hyacinth.

Isolation and identification of bacteria

The samples collected were serially diluted and plated on nutrient agar medium in a pour plate technique. Incubated for 24-48 hour at 30°C. Colonies that developed on agar were differentiated by color, elevation, form, and edge appearance. The representative bacterial colonies were picked up, purified by streaking on plates of nutrient agar. When a streaking produces only a single type of reproducible colony in a particular plate, it was considered to be pure culture and their colony characteristics were recorded. The purified isolates were transferred to nutrient agar slants in vials with a sterile loop, incubated at 37°C for 24-48 hours. After incubation, the vials were stored at 4°C and were maintained as stock culture.

Polyhydroxybutyrate (PHB) staining procedures and microscopy

There are various lipophilic dyes that have been used to stain polyhydroxybutyrate or fat inclusions inside the cells and to distinguish between PHB-accumulating and non-accumulating strains. In this study three such stains have been used: Nile red, Acridine orange and Sudan black B. All of them were obtained from Sigma-Aldrich, USA.

Rapid screening of isolates for PHB production by the Plate assay method

The ability to produce PHB was screened by growing isolates in Mineral Salt Medium containing $Na_2HPO_4.7H_2O$ 6.7 g; KH_2PO_4 1.5g; $(NH_4)_2SO_4$ 1 g; $MgSO_4.7H_2O$ 0.2g; Ferrous Ammonium Citrate 0.06g; $CaCl_2.2H_2O$ 0.01g and Glucose 5g (Ramsay et al. 1990). All the bacterial isolates were qualitatively tested for PHB production following the viable colony method of screening.

Fluorescent staining using Nile red: Screening of PHB using Nile red was performed according to the method of Spiekermann et al. (1999). Isolates of axenic colonies were randomly picked and cultured in solid mineral salt medium (MSM) as described by Ramsay et al. (1990). The sterilized mineral salt medium was supplemented with 0.5 mg Nile red stain (dissolved in 1ml dimethylsulfoxide) to give a final concentration of 0.5 µg dye per ml medium. The plates were exposed to ultraviolet light (360 nm) using a UV photometer after appropriate cultivation periods to detect accumulation of PHBs.

Screening with Sudan black B: All the bacterial isolates were qualitatively tested for PHB production following the viable colony method of screening using Sudan Black B dye (Juan et al. 1998). For rapid screening of PHB producers, nutrient agar medium was supplemented with 1 per cent glucose. The inoculated plates were incubated at 37°C for 24 hours. The ethanolic solution of (0.02%) Sudan Black B was spread over the colonies and the plates kept undisturbed for 30 minutes. Then the plates were washed with ethanol (96%) to remove the excess stain from the colonies. The dark blue coloured colonies were taken as positive for PHB production. The dark blue stained isolates were ranked in terms of '+' symbol.

Observation of granule formation

Observation using Acridine orange: Bacterial isolates were grown on mineral salt broth. For the preparation of smears with acridine orange 10 microliters of each 48-h-old culture was taken from the production medium and transferred to the Eppendorf tube, containing 50 microliter of acridine orange and incubated for 30 min at 30°C. After 30 minute the cultures were centrifuged at 10.000 rpm for 10 min. Then the pellets were collected and resuspended in distilled water according to Kumar & Prabakaran (2006). Smears were prepared on a clean microscopic slide and observed in a fluorescent microscope at 460 nm. The appearance of yellow colored granules inside the cell indicates the presence of PHB granules.

Observation using Sudan black B: This screening was carried out according to the method of Byrom & Byrom (1991). Isolates were grown on mineral salt media and smeared on a glass slide. A few drops of Sudan black solution was placed on the fixed preparation. After 5–10 minutes the ethanol in the stain should have evaporated. Any excess liquid can be carefully drawn off using the edge of a piece of filter paper. After that the slides were immersed on xylene until it is completely decolorized (this takes about 10 seconds) and allowed to dry. Then the slides were flooded with the counterstain safranine solution for 10 seconds. They were gently rinsed with running water and allowed to dry again. Then they were observed under an oil immersion lens. The PHB can be seen as dark blue granules inside red cells.

Identification of bacteria

The selected, most efficient PHB producing bacterial isolates were subjected to a set of morphological, physiological and biochemical tests for the purpose of identification. Gram staining was performed for all purified strains (Claus, 1995). Biochemical analyses were done by performing the catalase test, oxidase test, triple sugar iron reactions, citrate utilization test, urease test, indole test, motility test, Voges-Proskaur test, methyl red test and sugars fermentation test (Cheesbrough, 2001). Identification was compared with the Bergey's Manual of Determinative Bacteriology (Buchanan & Gibbons, 1994).

PHB production from glucose

PHB production was carried out in mineral salt medium (Ramsay et al. 1990) using glucose as a substrate. The bacteria were initially grown in nutrient agar medium for inoculum development. For shake flask experiments, a quantity of 20 ml medium was taken in 100 ml capacity Erlenmeyer flasks. They were inoculated with 10% (v/v) inoculums of overnight culture and incubated at 37°C, 120 rpm/min for 24-48 h. Samples at a different time interval (0, 24 and 48 hour) were spinned down at 4000 rpm for 10 minutes. Pellet was used for determination of cell growth whereas supernatant was used to determine reducing sugar content. PHB production was monitored by estimating the reducing sugar concentration in the fermentation medium. For glucose estimation, dinitrosalicylic acid method given by Miller (1959) was employed which is a simple, sensitive and adoptable method for handling of a large number of samples at a time.

Results and Discussion

In this study, attempts were made to find out polyhydroxybutyrate (PHB) producing bacteria from compost samples as compost is rich in microflora. Composting is a controlled, self-heating, aerobic solid phase biodegradative process of organic materials. It comprises of mesophilic and thermophilic phases involving numerous microorganisms. Under optimal conditions, the process can be divided into four phases involving numerous microorganisms. These are (i) an initial (first) mesophillic phase (10-42°C), which lasts for a few hours or a couple of days; (ii) a thermophilic phase (45-70°C), lasting a few days to several weeks or months; (iii) second mesophilic (cooling) phase and finally (iv) maturation stage which can last for several weeks to several months. The length of the different composting phases generally depends on the nature of the organic matter being composted and the efficiency of the process, which is determined by several factors such as starting material, O_2 supply, moisture content, active turning and outside temperature (Ryckeboer et al. 2003).

In this study, six types of compost samples were selected and only mesophilic bacteria (growing at 37°C) were isolated for further screening. The age of all compost samples ranged from a few weeks up to 4 months except bioslurry which was 6 months old. Poultry slurry 2 aged 2 months did not support any mesophilic bacterial growth. However, poultry slurry aged 3 months supported moderate microbial growth probably due to the completion of thermophilic phase and commencement of the cooling phase after 2 months. Table 1 shows a comparison of the microbial count of compost samples of the present study and other habitats found in previously reported literatures. In this study, bioslurry gave the highest mesophilic count compared to other compost samples and all the compost samples showed similar bacterial count as reported by others (Table 1).

Screening of the isolates for PHB production

The primary screening was performed using Nile red and Sudan black B stain. All 48 isolates were first grown on mineral salt medium supplemented with 5% glucose. From those 16 isolates were found to give orange fluorescence under UV (360 nm). Then they were further grown on a nutrient agar plate and tested with Sudan black. Based on the intensity of the fluorescence and Sudan black coloration, high PHB producers were identified. All isolates from poultry slurry 1 were PHB –ve. Shabaan et al. (2012) also employed Sudan black B and acridine orange staining for the screening of 180 soil isolates for PHB production. Figure 1 and Table 2 shows the result of screening using Nile red and Sudan black B. The isolates were further observed under a microscope using Nile blue, Sudan black and Acridine orange stain. Cells stained with Nile blue did not exhibit a fluorescence and therefore granules were not visible under fluorescent microscope. In Sudan black stained cells, PHB granules appeared as dark blue inside the red cells.

When acridine orange was applied to cells, PHB granules appeared as yellow granules under a fluorescent microscope.

Table 1. *Bacterial count in compost samples relative to other samples.*

Source	Microbial count (cfu/g)	Type	References
Cow dung (age<2 months)	3.25×10^7	Mesophilic aerobes	This study
Poultry slurry 1(3 months)	3.5×10^6	Mesophilic aerobes	This study
Bioslurry (6 months)	3.75×10^9	Mesophilic aerobes	This study
Tobacco dust (2 months)	3.0×10^7	Mesophilic aerobes	This study
Poultry slurry2 (2 months)	Nil		This study
Cotton Seed (4 months)	2.6×10^7	Mesophilic aerobes	This study
Thermogenic compost (<2 months)	10^7-10^{10}	Heterotrophic, gram-negative, rod-shaped, non spore forming, aerobic, thermophilic bacteria	Beffa et al. 1996
Microbial mat ecosystems	2.3×10^7	Heterotrophic	Berlanga et al. 2006
High-temperature synthetic food waste compost	2.6×10^8	Heterotrophic, thermophilic, aerobic	Dees & Ghiorse, 2001

Table 2. *List of PHB producing isolates after screening with Nile red and Sudan black B.*

Sample Description	Nile red positive strains	Fluorescence intensity	Coloration with Sudan black
Cow dung	CW16	+++	++
	CW10	++	++
	CW6	++	++++
	CW17	+++	+++
Bioslurry	BS16	+++	++++
	BS27	+	+
	BS7	+++	++
	BS2	++++	++
	BS23	++++	++
Tobacco dust	TD1	+	+
	TD3	++	+
	TD11	+++	++
Cotton seed	CS15	++	+
	CS14	++	+
	CS19	++	+
	CS18	++	++

Note: '+' poor, '++' medium, +++' strong, '++++' excellent

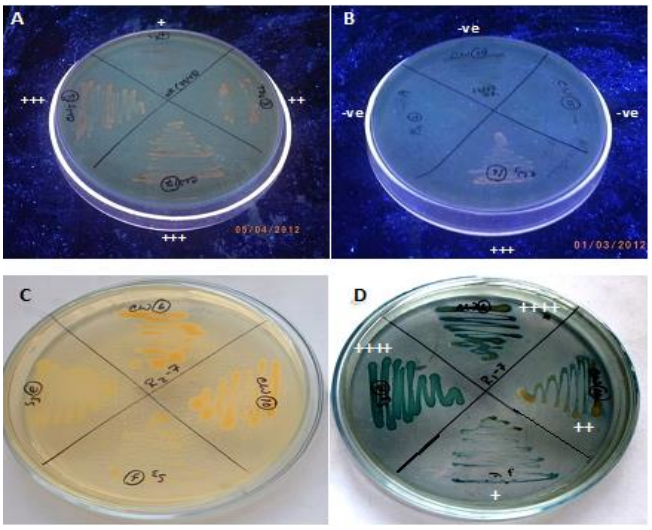

Figure 1. *Photographs showing (A,B) fluorescence of Nile red stained colonies under UV at 360 nm and comparison of (C) colonies before and (D) blue colored colonies appeared after application of Sudan black stains.*

Photomicrographs of Sudan black and acridine orange-stained cells are shown in Figure 2.

From fluorescence intensity and sudan black coloration, it was found that the maximum number of PHB producers were obtained from bioslurry sample. It also contained the isolates with maximum PHB accumulation. Tobacco dust contained the least number of PHB producing bacteria. Cow dung showed a good bacterial count with good PHB accumulation capacity and isolates from cotton seed showed moderate amount of PHB production capability.

Identification of the PHB producing isolates

A total of 16 PHB producing bacteria from different compost samples were screened from 48 isolates. Based on morphology, gram reaction and biochemical characteristics, all isolates were identified up to 'Genus' level by comparing with the standard strains already described in Bergey's Manual. As shown in Table 3, all isolates belonged to four

Table 3. *Provisional identification of the PHB producing isolates.*

Characteristics	BS2	BS23	CW17	BS7	BS16	CW16	TD11	CS18	CW10	TD3	CW6	CS15	CS14	CS19	BS27	TD1
Gram reaction	-	+	+	+	+	+	-	+	+	-	-	-	-	+	+	-
Shape	Rod	Cocci	Cocci	Rod	Rod	Cocci	Cocci	Rod	Cocci	Rod	Cocci	Cocci	Cocci	Rod	Rod	Rod
Catalase	+	+	+	-	+	+	+	-	+	+	+	+	+	+	-	+
Oxidase	+	-	-	-	+	-	-	+	–	+	-	-	-	-	-	+
NO_3 reduction	+	-	+	-	+	-	-	+	-	+	+	-	-	-	-	+
Indole	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-
MR	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
VP	+	+	-	+	+	+	-	-	-	-	+	-	-	+	-	-
Citrate	-	-	-	-	-	-	+	-	-	-	-	+	+	-	-	+
Spore staining					+			+						+		
Motility	-	+	+	-	+	-	+	-	-	+	+	-	-	-	-	-
O2 utilization	Facultative	Obligate aerobe	Facultative	Aerotolerant	Obligate aerobe	Facultative	Facultative	Obligate aerobe	Facultative	Obligate aerobe	Facultative	Obligate aerobe	Obligate aerobe	Obligate aerobe	Aerotolerant	Obligate aerobe
Hydrolysis of																
Starch	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
Gelatin	-	+	-	-	+	-	+	-	+	-	-	-	+	+	+	-
Casein	-	+	-	-	-	-	+	-	-	-	-	+	+	-	+	-
Acid production from																
Glucose	-	-	+	-	-	-	-	+	-	-	-	-	-	-	+	-
Sucrose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
Fructose	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-
Lactose	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-
Mannitol	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-
Arabinose	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-
Probable *genera*	*Pseudomonas*	*Micrococcus*	*Micrococcus*	*Bacillus*	*Bacillus*	*Micrococcus*	*Veillonella*	*Bacillus*	*Micrococcus*	*Pseudomonas*	*Veillonella*	*Veillonella*	*Veillonella*	*Bacillus*	*Bacillus*	*Pseudomonas*

genera viz. *Bacillus, Micrococcus, Veillonella* and *Pseudomonas*.

Out of 16 isolates, 5 isolates belonged to *Bacillus spp.*, four were *Micrococcus*, four strains belonged to the genus *Veillonella* and three strains belonged to *Pseudomonas as* shown in Table 3. In a similar approach, Aarthi and Ramana (2011) isolated five different species of *Bacillus* capable of producing PHB from garden soil. Shabaan et al. (2012) isolated strains of *Stenotrophomonas, Pseudomonas, Bacillus, Azotobacter, Azospirillum, Alcaligenes* from 15 different agricultural soil samples in Egypt.

Quantification of substrate consumption and cell growth

The economics of PHB production are largely determined by the substrate cost and PHB yield. So, the efficiency of substrate conversion is important. Among the various nutrients in the fermentation medium, the carbon source contributes most significantly to the overall substrate cost in PHB production. Glucose is one of the most convenient substrates used for PHB production for many microorganisms which costs around 0.493 US$/kg (Yamane, 1992).

In this study, glucose consumption and cell biomass were monitored in parallel during the fermentation. As shown in Table 4, six of the isolates were found with good glucose consumption rate and dry cell weight. These are BS2, CW17, BS7, CS18, CW10, and BS27. 24 hours old bacterial cultures were used for the observation of PHB production under a microscope. Out of 16 isolates, only six were found with good PHB accumulation which also showed better glucose

Table 4. *Change of cell dry weight and substrate consumption over time.*

Isolate Nos.	Cell Dry Weight (g/L)			Substrate Concentration (g/L)		
	0 hr	24 hr	48 hr	0 hr	24 hr	48 hr
BS2	4.3	3.6	4.0	4.95	1.49	0.97
BS23	4.5	5.1	3.6	4.99	2.80	2.11
CW17	3.9	5.6	2.5	4.89	3.11	1.77
BS7	4.95	4.8	2.1	5.00	3.43	2.39
BS16	5.4	4.2	2.6	4.97	2.91	2.11
CW16	4.6	5.0	3.0	4.88	2.47	2.27
TD11	4.05	4.6	2.7	4.90	2.96	1.63
CS18	4.95	5.6	3.1	4.79	2.76	2.18
CW10	3.9	4.5	3.7	5.02	3.11	2.28
TD3	2.1	4.2	2.9	5.01	3.25	3.02
CW6	2.9	4.6	3.6	4.99	2.81	2.13
CS15	3.0	6.1	4.8	4.67	1.99	1.66
CS14	3.8	4.3	4.0	4.78	2.15	1.56
CS19	3.1	4.3	2.8	4.91	3.71	1.89
BS27	1.8	4.5	4.3	4.69	2.80	2.11
TD1	2.8	4.4	4.4	5.00	3.18	2.73

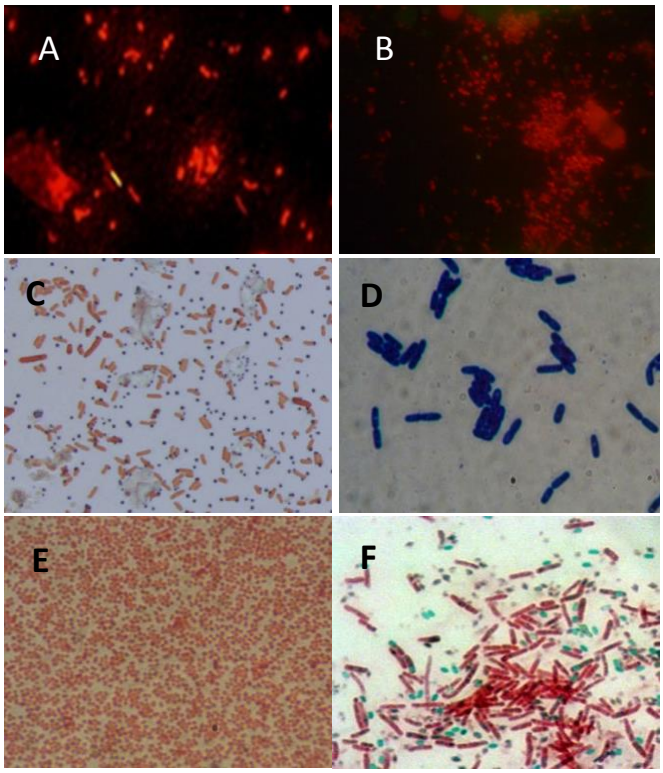

Figure 2. *Photomicrographs showing acridine orange stained cells of isolate no. (A) CS18 and (B) CS14 under an epifluorescence microscope; (C) Sudan black stained cells of isolate no. BS27; Gram staining of Isolate No. BS7(D) and CS14 (E) showing Gram positive and gram negative reaction, respectively; and spore staining cells of BS7 showing green colored spores, (indicated by arrow marks) with red vegetative cells. Under 100x magnification.*

consumption rate and CDW. These included three isolates of *Bacillus*, two isolates of *Micrococcus* and one isolate of *Pseudomonas sp*.

Conclusions

A total of 48 mesophilic isolates were obtained from different types of compost samples. Bioslurry showed the highest count (3.75×10^9 cfu/g) whereas the bacterial count of other samples e.g., cow dung, tobacco dust and cotton seed were 3.25×10^7 cfu/g, 3.0×10^7 cfu/g and 2.6×10^7 cfu/g, respectively. Sixteen potential poly-β-hydroxybutyrate (PHB) producing bacteria were successfully screened using Nile red and Sudan black. Acridine orange was used to visualize PHB granules under a fluorescent microscope. Maximum biomass and PHB accumulation were attained at 24-hour period from fermentation in shake flask using glucose as the carbon substrate. By analyzing photomicrographs of 24 hour old cultures, 6 out of 16 isolates showed good PHB accumulation capacity. These isolates belonged to four genera viz. *Bacillus*, *Pseudomona*, *Micrococcus* and *Veillonella*. These bacteria can be a potential source for commercial production of biopolymer and can also be tested for high PHB yield using other renewable substrates.

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