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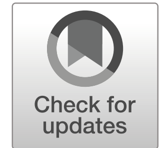

Hydrogenotrophic methanogen strain of *Methanospirillum* from anaerobic digester fed with agro-industrial waste

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Abstract

A hydrogenotrophic, mesophilic methanogen designated as strain $T_5$3BJ was isolated from anaerobic digester continuously fed with agro-industrial waste. Cells were stained Gram negative, spiral shaped, motile and found resistant to cell lysis by SDS and hypotonic solution. Growth occurred with optimum (i) temperature of 30 °C (20–40 °C) (ii) pH of 7.0 (6.5–8.5), in the presence of 0.0–0.2 M NaCl with a doubling time of 73.43 h and utilized only hydrogen and carbon dioxide (H_2:CO_2; 4:1) as a sole source of carbon and energy. The phenetic and phylogenetic analysis based on 16S rRNA and *mcrA* gene sequences revealed close affiliation of $T_5$3BJ strain to the genus *Methanospirillum* spp. This is the first report about isolation of hydrogenotrophic *Methanospirillum* spp. from anaerobic digester fed with fruit waste, press mud and neem seed cake, suggesting its potential use for bioaugmentation of anaerobic reactors or upgrading biogas/ syngas to methane.

Keywords Methanogens · *Methanospirillum* · Hydrogenotrophic methanogens · Anaerobic digestion · Agro-industrial waste

Abbreviations

AD	Anaerobic digestion
WL	Wood-Ljungdahl pathway
FW	Fruit waste
PMC	Press mud cake
NC	Neem cake
JISL	Jain Irrigation System Lmited
BCYT	Basal carbonate yeast extract and trypticase
PYG	Peptone yeast extract glucose
TCD	Thermal conductivity detector
SDS	Sodium dodecyl sulfate

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Introduction

Anaerobic digestion (AD) is an environmentally benign strategy to harness renewable green energy in the form of methane from the organic waste and responsible for the global carbon cycle (Gerardi 2003; Jha and Schmidt 2017; Robles et al. 2018). In anaerobic environments, the terminal step of the organic compound degradation to methane (Gerardi 2003) occurs in various natural and artificial habitats including, boreal wetlands, digestive tracts, aquatic sediments, geothermal vents, rice paddy fields, sewage sludge, anaerobic biogas digesters, etc. (Tonouchi 2002; Zhou et al. 2014; Jablonski et al. 2015;Bragulia et al. 2018; Hoelher et al. 2018), by a diverse group of syntrophic activity of anaerobic bacteria and methanogenic archaea through acetoclastic, methylotrophic and hydrogenotrophic methanogenesis pathway (Stams et al. 2012). Of these, hydrogenotrophic methanogens represent the most widespread pathway for ancestral methane production by class I and class II methanogens (Berghuis et al. 2019).

In anoxic environments, activity of hydrogenotrophic methanogens is crucial for the stability of methanogenesis because they (i) sequester hydrogen produced by microbial communities through interspecies hydrogen transfer (Stams 1994), (ii) metabolize CO_2 to methane by Wood-Ljungdahl (WL) pathway to generate energy using Mtr and Mcr protein, (iii) lower the partial pressure of hydrogen (<5.82 Pa) for

hydrogenase to function and (iv) allow stable acetogenesis resulting from the activity of syntrophic acetogens and acetoclastic methanogens to produce methane (Zábranská and Pokorna 2017). These hydrogenotrophic methanogens consume H_2: CO_2 and/or formate in anaerobic habitat and generally represent about 2/3 of total methanogenic population for 30% methanogenesis vis-à-vis 70% acetoclastic methanogenesis (Jablonski et al. 2015; Wagner et al. 2018). The most common members of hydrogenotrophic methanogens in anaerobic digesters belong to 5 orders such as, (i) *Methanoculleus*, (ii) *Methanospirillum*, (iii) *Methanoregula*, (iv) *Methanosphaerrula*, (v) *Methanobacterium*, (vi) *Methanobrevibacter* and (vii) *Methanothermobacter* (Sarmiento et al. 2011; Leng et al. 2017; Kouzuma et al. 2017) which reduce CO_2 in six steps via WL pathway to methane with H_2 as the reductant (Berghuis et al. 2019).

Currently, the genus *Methanospirillum* reported from various habitats comprises (i) two mesophilic methanogens namely, *Methanospirillum hungatei* (Ferry et al. 1974) and *Methanospirillum lacunae* (Iino et al. 2010) and (ii) two psychrophilic methanogens such as *Methanospirillum psychrodurum* (Zhou et al. 2014) and *Methanospirillum stamsii* (Parshina et al. 2014). Both mesophilic *Methanospirillum* grow at 30–37 °C, but unable to below 15–25 °C and utilize formate and H_2/CO_2 to produce methane (Tonouchi 2002; Iino et al. 2010), while psychrophilic *Methanospirillum* spp. grow at 4–35 °C (optimum at 25–30 °C) and utilize exclusively H_2:CO_2 (Parshina et al. 2014).

The present knowledge about ecology of methanogens from anaerobic digesters or allied habitats is obtained mainly from cultivation in the form of culture enrichment on a variety of substrate (Wette 2018) which has led to isolation of number of methanogens including, hydrogenotrophic isolates. However, isolation of methanogens in pure cultures is more challenging because of (i) long microbial growth enrichment times, (ii) sensitive to oxygen, (iii) dependence on syntrophic bacterial association and (iv) limited diversification in cultivation strategies. It is even more difficult in case of isolation of hydrogenotrophic *Methanospirillum* from anaerobic digesters since critical requirement for H_2 and CO_2. Moreover, *Methanospirillum* are important hydrogen scavenging methanogen in VFA degrading cocultures and hence, pure culture of *Methanospirillum* may have potential for bioaugmentation to improve the efficiency of anaerobic digesters and understand microbial interactions leading to bio-methanogenesis (Zábranská and Pokorna 2017). For this purpose, electrochemical bioreactor (Jeon et al. 2009) and gas recycle approach (Yun et al. 2017) were reported for the enrichment of hydrogenotrophic methanogens. At present, four strains of *Methanospirillum* spp. were isolated using Hungate anaerobic technique (1969). While hydrogenotrophic methanogen from hot spring (Joshi et al. 2018) and rice paddy field were reported earlier (Adachi 1999; Tonouchi 2002). Similarly, the predominance of hydrogenotrophic *Methanospirillum hungatei* was reported earlier from biogas reactors containing liquid cattle manure (70%) and liquid pig manure (30%) with dry mass of 3–4% consecutively fed with maize silage and bruised grain for 33 days at 36–38 °C using RT PCR with primers targeting the *mcrA* gene (Lin et al. 2009; Kampmann et al. 2012). Of total 198 methanogens, only 28 strains have been obtained from anaerobic digesters, but no *Methanospirillum* have been reported yet from digesters operated with agro-waste such as fruit waste (FW), press mud cake (PMC) and neem seed cake (NC). Hence, the present study attempts to isolate, purify and characterize hydrogenotrophic methanogen from anaerobic digester fed with agro-industrial waste.

Materials and methods

Source of inoculum for isolation of methanogens

The mesophilic anaerobic Bunglow digester (D1T5; 10 m^3) operated for six years on organic feedstock such as neem seed cake (NC) and press mud cake (PMC; a sugar industry waste) at 37 °C was used as a source of inoculum for the isolation of methanogen(s). The inoculum for the digester was digestate slurry from a mesophilic digester operated at Jain Irrigation Systems Limited (JISL), Jalgaon, India and fed with similar type of feed material. Digestate inoculum (60 mL) from anaerobic digester was collected in 65 mL pre-sterile glass bottle. Before sampling, the glass serum bottles were flushed with O_2 free N_2 gas @ 15 psi pressure for 5 min, then secured with butyl rubber stopper and finally crimped with aluminum seal.

Media and cultivation conditions for enrichment and isolation of methanogen strains

The enrichment and isolation of methanogens was performed in 65 mL glass serum bottles. (Tonouchi 2002). For this purpose, about 2.0 mL aliquots of digestate inoculum was inoculated into serum bottles containing 20 mL basal carbonate yeast extract and trypticase (BCYT; pH 7.0) with H_2:CO_2 (80:20, *v*/v) as a substrate (Ferry et al. 1974; Iino et al. 2010; Parshina et al. 2014) and each bottle was sealed with a tight fitting butyl rubber stopper and aluminum cap. Each bottle was continuously flushed with oxygen free H_2:CO_2(80:20, *v*/v) gas at 15 psi pressure using gassing manifold. All inoculated bottles (22 mL) were incubated at 35 °C. The experiment was performed in three sets with two negative controls (one without inoculum and another without substrate, H_2: CO_2 but with inoculum). The details of inoculation scheme are given in Table 1 (Supplementary data). BCYT (20 mL in each bottle) medium used for the isolation of methanogens was prepared as per Touzel and Albagnac (1983). The basal medium used was composed of (gL^{-1}) NH_4Cl, 1.0; NaCl, 0.6; Yeast extract,

0.5; Trypticase, 0.5; KH_2PO_4, 0.3; $MgCl_2.6H_2O$, 0.1 and $CaCl_2$ (anhydrous), 0.08; Reducing agent (Ranade and Gadre 1988), 1 mL trace elements (Wolin et al. 1963). Each ingredient of the basal medium was dissolved in preboiled distilled water and then, flushed with N_2 gas at15 psi pressure. The pH of the medium was adjusted to 7.0 ± 0.2 with 0.1 N NaOH or 0.1 N HCl prior to inoculation. The medium was again flushed with nitrogen gas using gassing manifold. In each 65 mL serum bottle, about 20 mL of medium was dispensed (Hungate 1950), stoppared with butyl rubber stopper, sealed with aluminum seal, sterilized at 121 °C for 15 min at 15 psi, cooled, flushed with N_2 gas, then supplemented with 1 mL filter sterilized vitamin (Wolin et al. 1963) from fresh stock solution and flushed again with O_2 free N_2 gas. For the growth of non- methanogens, Peptone yeast extract glucose (PYG) medium (Holdeman et al. 1977), Mc medium (Borrel et al. 2012) and glucose rich nutrient broth (Jones et al. 1983) containing Resazurin (1 mgL^{-1}) were prepared anaerobically under a gas atmosphere of 99.999% N_2 using the Hungate anaerobic technique (Macy et al. 1972). The glucose rich nutrient broth was prepared aerobically to check the growth of aerobic bacterial contaminants, if any.

Selection and transfer of enrichment

Methane released in the headspace of each serum bottle was detected after every 8 days during enrichment transfer phase with gas chromatograph (Nucon 5765, India) as per Suryawanshi et al. (2009). Each bottle was pre-flushed with H_2: CO_2 (80:20, *v*/v) at 15 psi pressure on every alternate day during the incubation period. The bottles showing more than 30% of methane in the headspace were selected for further transfer in fresh BCYT medium containing H_2: CO_2 (80:20, v/v) as a substrate.

Methane analysis

The serum bottles were analyzed for the growth of methanogen(s) with respect to methane released in the head space after incubation. Methane content in each serum bottle was analyzed by gas chromatograph (Nucon 5765, India) using thermal conductivity detector (TCD) (Suryawanshi et al. 2009) and stainless steel (SS) column packed with Porapak Q (80/100 mesh range, 2 m length and 1/8″ diameter). The flow rate of carrier Helium gas was 10 $mLmin^{-1}$. The oven, injector and detector temperatures were kept at 60, 75 and 200 °C, respectively. The biogas sample was taken from the headspace of serum bottle with the help of gas tight syringe. The surface head of each serum bottle and needle of the syringe were cleaned with alcohol before taking biogas sample for analysis. The sample was then injected to the injector of gas chromatograph. NuChrom software was used for data interpretation. A standard methane canister (99.99% purity from Alchemie gases, Tarapur, India) was used to standardize the methane estimation.

Isolation and purification of methanogen

The positive methanogenic enrichments showing the presence of non-methanogenic bacterial contaminants were subjected to three stage successive transfer to fresh BCYT broth at 8 days interval using, (i) serial dilution method (Balch et al. 1979), (ii) treatment with antibiotic (Kadam 1988) and (iii) roll tube (Hungate 1950, 1969). In serial dilution method, the highest decimal dilution (10^{-5}) showing the presence of methanogens was inoculated in the BCYT medium containing H_2:CO_2 (80:20, *v*/v)) and incubated at 35 °C for a period of 30 days. The antibiotic sensitivity assay of bacterial contaminants was performed with a purpose to reduce their load during the successive enrichments as per the method of Borrel et al. (2012). For further purification of methanogenic isolate, roll tube method described by Hungate (1950) and Hungate (1969) was used. Last two decimal dilutions showing the presence of methanogens were used for roll tube and incubation was continued until visible isolated colonies were observed. After the typical visible colonial growth, gas sample in the head space of the roll tubes were analyzed for methane content and the roll tubes showing maximum methane content were selected for the colony transfer.

Colony transfer, re-roll tube and confirmation of purity of the hydrogenotrophic methanogens

Typical colonies from roll tubes were inoculated in the serum bottles containing BCYT medium (H_2:CO_2, 80:20, *v*/v) and incubated at 35 °C for a period of 20 days. The purity of the methanogen isolate was confirmed by re-isolation using re-roll tube. The absence of non-methanogens was ensured using phase contrast microscopy and the persistence of non-methanogenic bacterial growth was confirmed with three separate growth media devoid of methanogenic substrates namely, (i) PYG medium (Holdeman et al. 1977), (ii) Mc medium (Borrel et al. 2012) and (iii) glucose rich nutrient broth (Jones et al. 1983). Each medium was inoculated with the culture for the presence of non-methanogens, if present and incubated at 35 °C for a period of 7 days.

Phenetic identity

Morphological characterization of the isolated methanogen was carried out as per Boone and Whitman (1988) using (a) phase contrast microscopy and (b) epi-fluorescence microscopy.

The methanogen isolate was investigated for the (i) growth at pH (5–9), temperature (10–50 °C), and (ii) requirement of sodium chloride (0 -1 M) using H_2:CO_2 (80:20, *v*/v) as a substrate as per Boone and Whitman (1988). The isolate was

also determined for growth on thirteen different substrates. Except H_2:CO_2, all substrate were added at the final concentration of 30 mM. Each biochemical test was performed in triplicate by inoculating pre-grown culture at optimal conditions for specified period and observed for methane production. Production of methane in the headspace of serum bottle was used to evaluate optimum growth requirement of the methanogen(s). Methane produced (%) during the incubation period for each parameter was determined.

The specific growth rate (μh^{-1}) of methanogen isolate was determined from the concentration of CH_4 formed during the logarithmic growth phase as per Powell (1983). Susceptibility of cells to sodium dodecyl sulfate (SDS) lysis was determined by dispensing 2 mL aliquots of a freshly grown methanogen isolate (Boone and Whitman 1988).

Molecular characterization

The genomic DNA of methanogen isolate was extracted and purified using QIAamp® DNA tool kit (Qiagen, Germany) and PCR amplified 16S rRNA and *mcr*A gene using (A10F and A1400R) primers: A10F (5’ TCY GGT TGA TCC YGC CRG-3′), A1400R (5’ ACG GGC GGT GGT GCA AG-3′), *mcr*A-F (5′-GGT GGT GTM GGA TTC ACA CAR TAY GCW ACA GC-3′) and *mcr*A-R (5’-TTC ATT GCR TAG TTW GGR TAG TT-3′), respectively were carried out at Xcelris Genomic Centre, Ahmadabad. The reaction mixture (25 μL) was comprised of 10X Taq buffer, dNTPs (10 mM each), 2X PCR master mix (MBI fermentas), primers (10 pmol/μL each) and nuclease-free water. The steps and conditions of thermal cycling for Met A10F / A1400R and *mcrA*-F / *mcrA*-R targeting 16S rRNA and *mcrA* genes, were carried out according to Wright et al. (2004) and Luton et al. (2002), respectively. The amplified PCR products were purified and sequenced using QIAquick Gel extraction kit (Qiagen, Germany) and BigDye Terminator v3.1 cycle sequencing kit, respectively at Xcelris Genomic Centre, Ahmedabad. Sequences were checked for quality and contigs were prepared before BLAST search. The sequences were obtained to find out the homology with published methanogen sequences available in GenBank database.

Phylogenetic analyses were conducted in MEGA 5.0 (Tamura et al. 2011) using 16S rRNA sequences of methanogens from NCBI GenBank database. Phylogeny was further confirmed by *mcrA* protein sequences.

Results and discussion

Selection and transfer of enrichment

The enrichment culture (D1T5) in the presence of H_2: CO_2 (80:20; *v*/v) at 35 °C after 20 days showed methane productivity of 33, 35, 36, 36, 38, 42, 42 and 46% in the head space of serum bottles during enrichment transfer (Table 1). After seventh enrichment to BCYT medium containing H_2: CO_2, (80:20; v/v), the broth culture was observed for the prevalence of the cellular morphotypes since serum bottles showed 46% methane in the headspace. The microscopic observation of the enrichment (7th) showed the presence of spiral shaped methanogens along with 6–8 cells of nonmethanogen bacterial cells per microscopic field.

Selection and transfer of enrichments on the ‘theory of selection and elimination’ was carried out on the basis of (i) more than 30% methane content (33–46%) in the headspace, (ii) negligible density of non-methanogens detected under phase contrast microscopy and (iii) higher density of autofluorescent spiral rods of methanogens under florescence microscopy predominantly resembling *Methanospirillum*. The similar observation about predominance of hydrogenotrophic *Methanospirillum* like cells was earlier obtained in a mesophilic full-scale biogas digester treating pig manure as well as maize silage, different liquid manures, and renewable raw materials (Rastogi et al. 2008; Bergmann et al. 2010; Kampmann et al. 2012). In another study, Tonouchi (2002) also reported prevalence of hydrogenotrophic *Methanospirillum* from paddy fields. However, predominant growth of *Methanospirillum* like methanogens in the enrichment culture was unexpected because all our previous studies revealed enrichment and isolation of hydrogenotrophic acetoclastic methanogens from mesophilic anaerobic digesters led to isolation of *Methanosarcina* (Suryawanshi 2012). Previous studies also demonstrated more hydrogenotrophic *Methanosarcina* (Demirel and Scherer 2008). Accordingly, particular enrichment from digester (D1T5) was selected from seven transfers for further isolation and purification of methanogens based on methane released (46%) in the headspace of serum bottle.

Isolation and purification

The selected enrichment (7th) was successively transferred twice in BCYT medium at same dilution (10^{-4}) which showed the persistence of 5–6 non-methanogens. Hence, antibiotics (penicillin, kanamycin, cephalothin, clindamycin, ampicillin, vancomycin, chloramphenicol and D-cycloserine) at final concentration of 100 $\mu g mL^{-1}$ (except D-cycloserine at 10 $\mu g mL^{-1}$) were applied to selected enrichment in order to eliminate the non-methanogenic bacterial population.

For this purpose, antibiogram profile of each non-methanogenic isolate was carried out in PYG medium at 35 °C. The preliminary antibiotic assay indicated sensitivity of non-methanogens to cephalothin and vancomycin. Hence, gentamycin (100 $\mu g mL^{-1}$) and vancomycin ($\mu g mL^{-1}$) were applied to further transfer of methanogenic enrichment. Earlier, Zhou et al. (2014) used serially diluted basal medium

Table 1 Profile of successive transfers of enrichments with respect to % methane content in the headspace of glass serum bottles analyzed by gas chromatography

Digester	H_2: CO_2 80:20 (v/v)	Methane (%)		
		Set I	Set II	Set III
D1 (T5)	Inoculation (2 mL)	30	33	28
	I	32	35	32
	II	30	36	30
	III	28	36	26
	IV	26	38	22
	V	19	42	20
	VI	–	42	16
	VII	–	46	–
Control (Negative)	Without inoculum	–	–	–
Control (Negative)	Without H_2: CO_2 but with inoculum	–	–	–

containing penicillin (0.5 gL^{-1}) and kanamycin (0.5 gL^{-1}) for the isolation of *Methanospirillum psychrodurum* from wetland soil. For the isolation of number of methanogens, Whitman et al. (2006) have employed treatment with various antibiotics such as ampicillin, kanamycin and vancomycin and in case of *Methanospirillum stamsii*, ampicillin (1gL^{-1}), penicillin (2 gL^{-1}), vancomycin (100–200 mgL^{-1}), cycloserine (100 mgL^{-1}), kanamycin (100 mgL^{-1}), erythromycin (100 mgL^{-1}) or rifampicin (100–200 mgL^{-1}) were used (Parshina et al. 2014). After four successive transfers in the present study, the load of non-methanogens was significantly reduced with distinct blue-green auto-fluorescence spiral shaped morphotypes under UV light in fluorescence microscope, suggesting the presence of methanogens.

Several antibiotics either in single or in combination have been used earlier for the purification of methanogens (Jeanthon et al. 1999; Lai et al. 2002; L'Haridon et al. 2003; Shlimon et al. 2004). Wildgruber et al. (1982) used MG medium containing penicillin, vancomycin, kanamycin and tetracycline to avoid eubacterial contamination for the isolation of *Methanoplanuslimicola*. The penicillin at concentration in the range of 50–200 mgL^{-1} was used in the isolation of *Methanosarcina baltica* (von Klein et al. 2002).

Each serum bottle showed the absence of non-methanogens and the presence of only one morphological form of methanogen under microscope. The purity of methanogen for the complete elimination of non-methanogens was finally ensured in PYG broth after prolong incubation of 15 days.

Further isolation of methanogens was made by roll tube method using BCYT medium (H_2: CO_2; 80:20, *v*/v). After incubation of 7 days at 35 °C, pin point colonies were observed in roll tubes. Each roll tube was further incubated for a period of 10 days till isolated single morphotype colonies appeared on the surface of medium. Isolated colonies (Bergmann et al. 2010) were transferred to serum bottles. Of these, only single serum bottle exhibited almost 40% of methane after 20 days of incubation, while remaining three bottles showed 25–30% of methane even after 30 days of incubation. The serum bottle that produced almost 40% methane and showed spiral shaped methanogens under phase contrast as well as fluorescence microscope was selected for further transfer. The re-inoculation in roll tube confirmed its purity. The typical morphology of the colonies appeared in the re-roll tube were similar to that observed in previous roll tube.

Until now, only Lin et al. (2009) and Kampmann et al. (2012) have found *Methanospirillum hungatei* related species as the most dominant hydrogenotrophic methanogens in biogas reactor fed with pig manure and liquid manure. Later, Yun et al. (2017) reported relative abundance of hydrogenotrophic *Methanospirillum* in mesophilic reactor employing gas recycle.

The pure culture of methanogenic isolates obtained in this study using three stage purification protocols was designated as $T_5$3BJ. The purity of isolate was confirmed for the absence of (i) aerobic growth of nonmethanogenic bacterial contaminants in nutrient broth containing 1% glucose, and (ii) anaerobic bacterial contamination in PYG broth and Mc medium. Similar strategy was used earlier (Yeole 1986); but, Borrel et al. (2012) have suggested Mc medium for purity check of *Methanobacterium lacus*.

Morphological characteristics

Isolate $T_5$3BJ formed diffuse, whitish, irregular colony with 0.5–1 mm diameter, convex with entire margins on BCYT agar after 30 days of cultivation under H_2: CO_2 (80:20,*v*/v). Individual cells of isolate were spiral shaped, occasionally wavy, motile, stained Gram negative, non-spore forming rods and cells appeared blue-green auto-fluorescence under UV light under fluorescence microscopy, indicating the prevalence of coenzyme F420. The phenotypic observations of strain $T_5$3BJ cells agreed with the member of genus

Methanospirillum spp. (Ferry et al. 1974). Phase contrast and epi-fluorescence images of the isolate $T_5$3BJ are as shown in Fig. 1.

Optimal pH for methanogenesis

It is seen that the isolate $T_5$3BJ was unable to grow pH 6.5 and above 8.0, as there was no methane production, but maximum methane production (18.06%) was observed at pH 7.0, followed by 7.5 and 8.0 (Fig. 2). The results are in line with Tonouchi (2002). However, absolute methane production was almost negligible at pH 6.0 and 8.5. Hence, the pH range for methanogenesis was 6.5–8.0 and the optimum pH of the isolate was 7.0 on the basis of maximum average methane (18.06%). The results of $T_5$3BJ isolates are in observations with Ferry et al. (1974), who recorded optimum pH range of 6.6 to 7.4 for *Methanospirillum hungatei*. Whereas, *Methanospirillum lacunae*, *Methanospirillum stamsii* and *Methanospirillum psychrodurum* grew optimally at pH 7.5, 7.0–7.5 and 7.0, respectively for methane production (Iino et al. 2010; Parshina et al. 2014; Zhou et al. 2014).

Optimum temperature for methanogenesis

Isolate $T_5$3BJ was able to grow between the temperature ranges of 20–40 °C. The significant methane production was obtained at 30 °C (19.17%) followed by 35 and 40 °C (Fig. 3). This observation appears to be in accordance with the anaerobic digester operated under steady state at mesophilic temperature range when fed with PMC and NC. Therefore, the maximum activity of the isolates was expected in the mesophilic temperature range. These results are in accordance with Tonouchi (2002). These findings are compared with previously reported optimum temperature of 30 °C for *Methanospirillum lacunae* and are found lower than 30–37 °C for *Methanospirillum hungatei* JF1 (Iino et al. 2010; Parshina et al. 2014). Another strain of *Methanospirillum hungatei* GP1 has an even higher temperature optimum at 45 °C (Patel et al. 1976). Whereas, *Methanospirillum stamsii* showed optimum methanogenesis in the temperature range of 20–30 °C and *Methanospirillum psychrodurum* grew optimally at 25 °C (Zhou et al. 2014; Parshina et al. 2014).

Optimal NaCl for methanogenesis

The requirement of NaCl for the growth of isolate $T_5$3BJ was examined in BCYT medium at various concentrations in the range of 0.1–1.25 M at 30 °C (pH 7.0). It is clear from Fig. 4 that the isolate was able to tolerate the range of salt concentration from 0.0 to 0.2 M, but inhibited above 0.2 M. Maximum methane production (18.44%) was noticed in the absence of salt. It is noteworthy that the isolate was able to grow in the absence of salt. The findings are in agreement with *Methanospirillum stamsii* which also displayed growth and methane production in the absence of NaCl (Parshina et al. 2014). However, a significant drop in methanogenesis was observed at and above 0.3 M NaCl. Interestingly, sodium salt is essential on one side for ATP production and oxidation of NADH, but excess concentration proved detrimental and upset microbial metabolic activity (Dimroth and Thomer 1989).The results in the present study are in line with earlier findings of Iino et al. (2010) who observed optimal growth of *Methanospirillum lacunae* in the absence of sodium salt in the basal medium.

Substrate utilization spectrum

Methanogens are highly sensitive and selective about substrate for growth, survival and methane production. Generally, almost all methanogens are limited to single carbon compounds such as H_2: CO_2 and acetate as preferred substrates. Due to this limitation, methanogens are often dependent on syntrophic bacteria to degrade higher compounds to corresponding simpler utilizable forms. For this reason, substrate specificity is a highly critical factor for the determination of the type of methanogen and corresponding habitat.

It was observed that the isolate $T_5$3BJ was able to utilize only H_2: CO_2 for methane production, whereas, it was unable

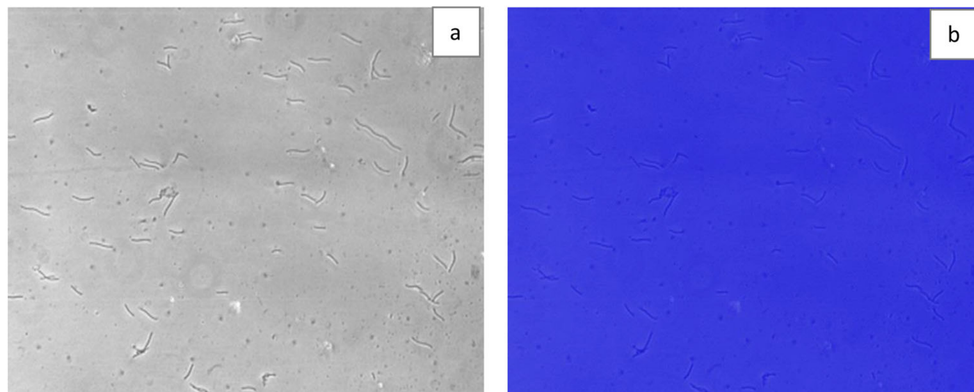

Fig. 1 Phase contrast (**a**) and fluorescence microphotograph (**b**) of the methanogen strain $T_5$3BJ isolated after enrichment from anaerobic digester

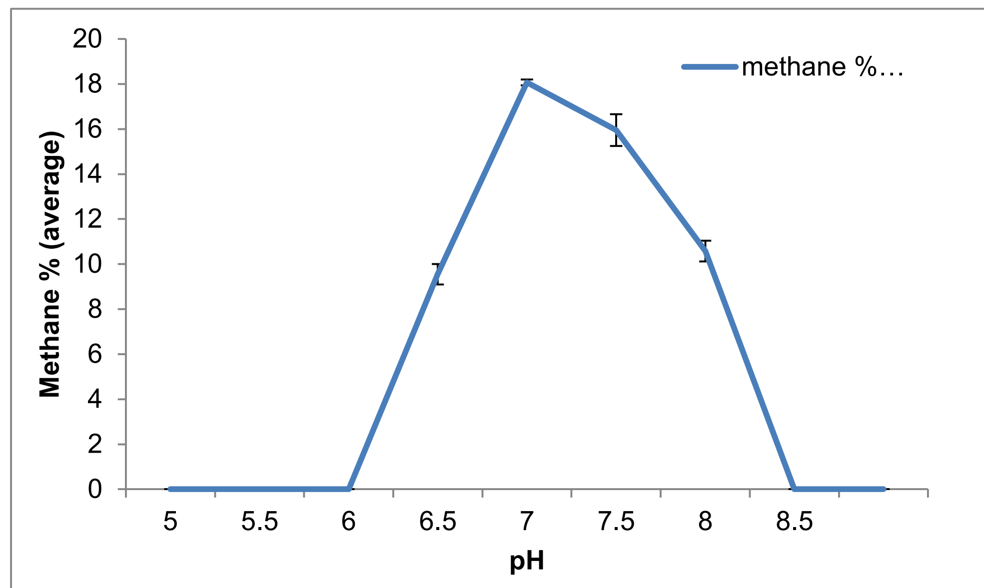

Fig. 2 Effect of pH on the growth of methanogen isolate $T_5$3BJ for methanogenesis at 35 °C

to use other substrates such as formate, acetate, dimethylsulphide, methylamines, methanol, 1-propanol, 2-propanol, 1-butanol, 2-butanol, cyclopentanol for methane production, indicating that isolate $T_5$3BJ is hydrogenotrophic methanogen. Isolate $T_5$3BJ produce 18.78% average methane using H_2: CO_2 (80:20, *v*/v) as a substrate. The present findings are in agreement with Zhou et al. (2014) who have observed that *Methanospirillum psychrodurum* utilize only H_2: CO_2 for methane formation and acetate could not induce its growth. Whereas, *Methanospirillum hungatei* and *Methanospirillum lacunae* can utilize both substrates, H_2: CO_2 and formate for methane production and 4 strains of *Methanospirillum* were unable to use rest of substrates. In contrast, Iino et al. (2010) showed that acetate and yeast extract were indispensible for the growth of *Methanospirillum lacunae* and *Methanospirillum hungatei* JF^{-1} (Zhou et al. 2014). In another study, hydrogenotrophic *Methanospirillum* TM20–1 not only utilize H_2: CO_2, but also catabolize 2-propanol: CO_2 or formate as substrate and acetate was required for the growth (Tonouchi 2002). While, Patel et al. (1976) have demonstrated that *M. hungatei* GP-1 from AD fed with pear waste utilize acetate in the presence of H_2. However, Adachi (1999) have found 52% methanogenic isolates from paddy field utilize H_2: CO_2 as sole source for energy and carbon for growth, 48% utilized formate in addition to H_2: CO_2 and unable to utilize methanol and acetate. Interestingly, another study demonstrated more abundance of *Methanospirillum hungatei* in undisturbed and bioaugmented digester fed with non-fat dry milk compared to the presence of more *Methanolinea tarda* in upset and non-bioaugmented digesters (Tale et al. 2015).

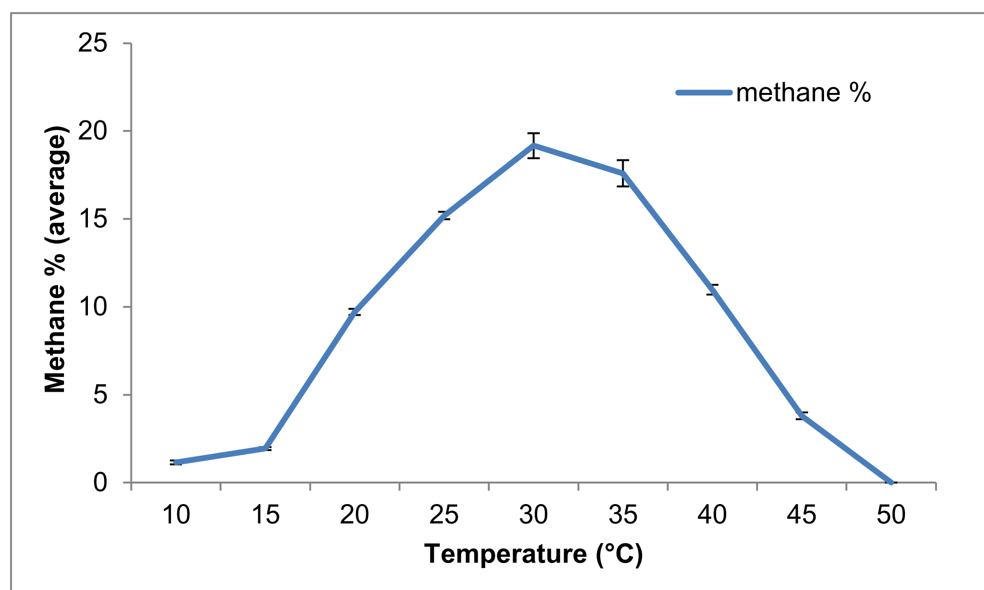

Fig. 3 The methanogenesis of isolate $T_5$3BJ at various temperatures in BCYT medium

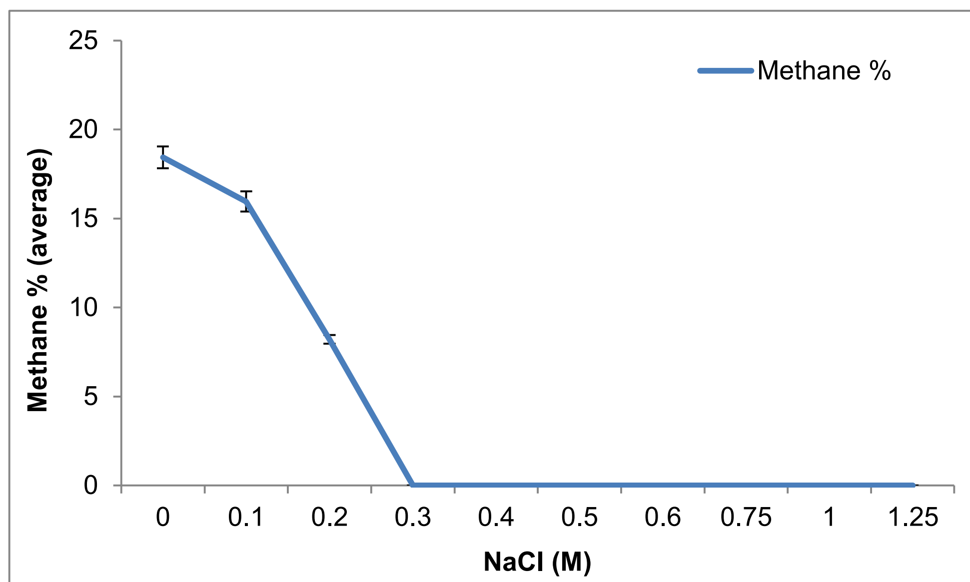

Fig. 4 Effect of salt concentration on methanogen isolate $T_5$3BJ for methanogenesis at 30 °C in BCYT medium (pH 7.0)

Specific growth rate (μh^{-1}), generation time (h) and cell lysis

The measurement of specific growth rate of isolate $T_5$3BJ was done by methane production (%) using H_2: CO_2 (80:20, *v*/v) as a substrate at optimum condition of pH (7.0), temperature (30 °C) and NaCl (0.0 M). The specific growth rate of isolate $T_5$3BJ was 0.0094 μh^{-1} and generation time of 73.43 h, indicating that hydrogenotrophic strain possibly monitoring H_2 in anaerobic digester fed with PMC and NC. In contrast, *Methanospirillum stamsii* Pt1 showed doubling time of 39.8 h in the medium supplied with H_2: CO_2 at 30 °C and pH 7.5 (Parshina et al. 2014) and *Methanospirillum psychrodurum* revealed specific growth of 0.065 h^{-1} in basal medium under H_2: CO_2 at pH 7 and 25 °C (Zhou et al. 2014). Whereas, *M. lacunae* Ki-8 and *M. hungatei* NBRC 100397 showed variable generation time of 32.3 h and 20.7 h, respectively at 40 °C in HAB medium (pH 7.5) under H_2: CO_2 (4:1, *v*/v) (Iino et al. 2010).

Cell lysis

The pelleted aggregate of isolate showed no further increase in turbidity after exposure to 1.5% SDS or distilled water for 10 min. Even microscopic observations also revealed intact cells after 5 h of SDS treatment. Thus, the isolate was found to be resistant to lysis by SDS and hypotonic solution. The findings are in agreement with results reported by Parshina et al. (2014) with the observations that pelleted cells of *Methanospirillum stamsii* were resistant to lysis by 2% SDS. Similar observations were reported with *M. psychrodurum* strain X-18 (Zhou et al. 2014) and *Methanospirillum* TM 20–1 (Tonouchi 2002).

Molecular characterization

16S rRNA gene sequence of 1343 bp was obtained from the isolate $T_5$3BJ (Fig. 1a Supplementary data). The identities of isolate were uncovered by comparing 16S rRNA gene sequences using BLAST similarity searches against data base entries. 16S rRNA gene sequence of isolate was submitted to NCBI gene bank (MH712280). The strain $T_5$3BJ was closely affiliated with known species of the genus *Methanospirillum* and most closely with *Methanospirillum stamsii* Pt1. Methanogenic isolate $T_5$3BJ shared a high degree of 16S rRNA gene sequence similarity (99%) with *Methanospirillum stamsii* strain Pt1 (Fig. 5).

The *mcrA* gene sequence of 783 bp was obtained (Fig. 1b Supplementary data) from the isolate $T_5$3BJ and partially amplified by PCR with primers. The *mcrA* gene sequences of strain $T_5$3BJ (MT551924) was aligned together with available sequences from NCBI database and maximum likelihood phylogenetic tree was reconstructed using MEGA 5.0 software clustered with the expected reference sequences (Fig. 6) and clustered with *M. stamsii* Pt1 (99%). Overall, 16S rRNA and *mcrA* gene tree topology resembled each other, thereby validating the identity of *Methanospirillum* spp. Accordingly, the hydrogenotrophic methanogen $T_5$3BJ belongs to *Methanospirillum* strain.

The comparative study of the isolated strain with previously isolated strains of genus *Methanospirillum* is shown in Table 2f. The morphological, physiological and biochemical characteristics differentiate the isolate $T_5$3BJ from all four reported strains of *Methanospirillum*. The strain $T_5$3BJ was isolated from anaerobic digester fed with PMC and NC operated at mesophilic temperature.

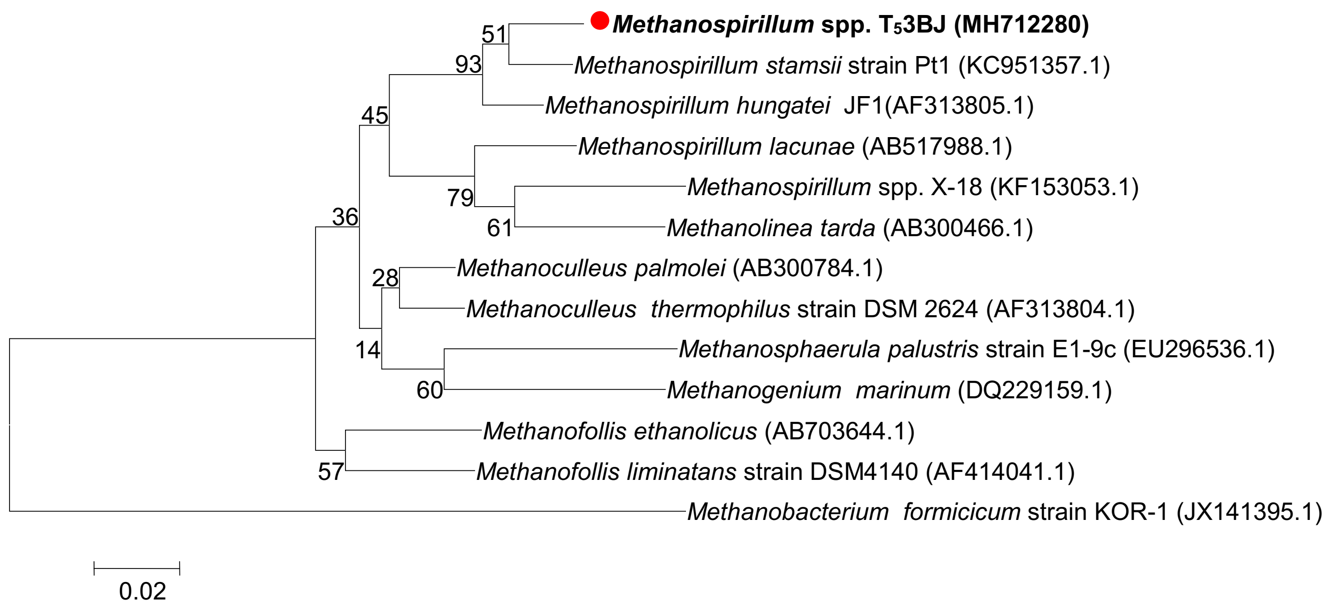

Fig. 5 A phylogenetic tree of hydrogenotrophic methanogen isolate $T_5$3BJ based on 16S rRNA gene sequence similarity with related strain from GenBank. The Gene Bank accession numbers for reference sequences are given in parentheses. Each 16S rRNA gene sequences were aligned with Clustal W and compared using MEGA 5.0 software. The evolutionary history of isolate was inferred using neighbor-joining (N-J) method and boot strap re-samples 500 times. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site (0.02 substitution per nucleotide position)

The bioaugmentation of the isolated strain can improve the digester efficiency with high VFA as hydrogenotrophic methanogens helps to keep low H_2 partial pressure required for syntrophic bacteria for VFA degradation. Bio-augmentation of pure culture of *Methanospirillum* spp. may also help to understand the microbial interactions and ecology in the hydrogenotrophic methanogenesis process.

Conclusion

The results presented imply new mesophilic hydrogenotrophic methanogen strain of $T_5$3BJ isolated from anaerobic digesters fed with agro-industrial waste operated at pilot scale (10 m^3). However, no such studies have been carried out earlier from the digester fed with PMC and NC. On the basis of phenetic

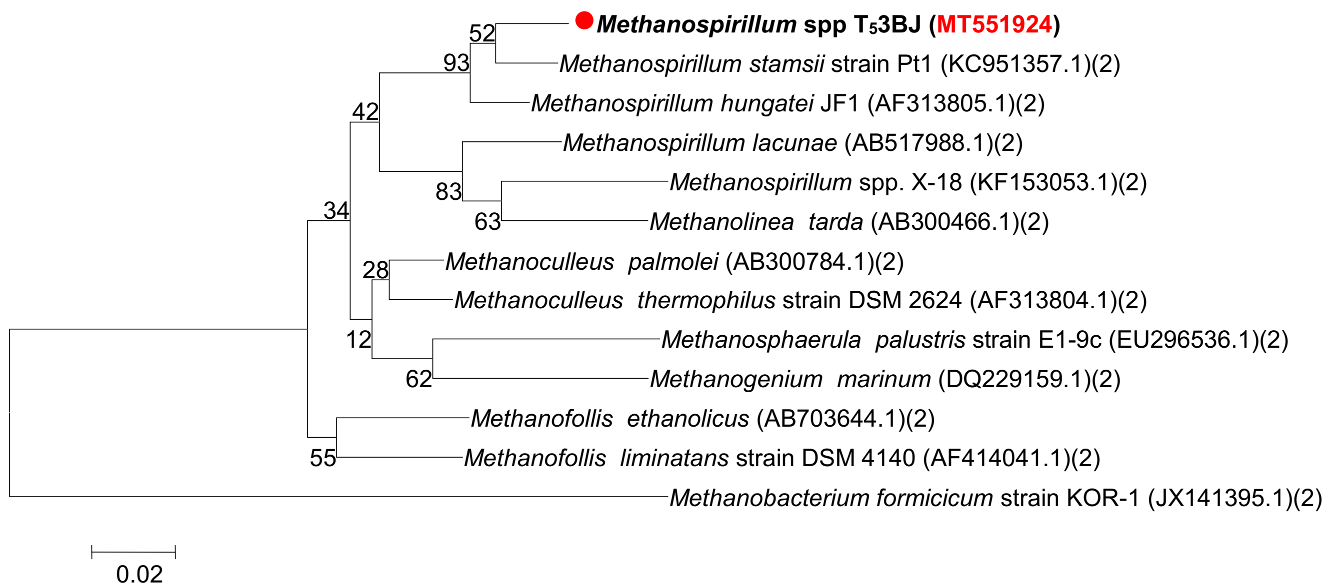

Fig. 6 A phylogenetic tree of *mcrA* amino acid sequences from methanogen isolate $T_5$3BJ based on *mcrA* partial gene sequence (783 bp) similarity isolated from anaerobic digester. The Gene Bank accession numbers for reference sequences are given in parentheses. Each *mcrA* gene sequences were aligned with Clustal W and compared using MEGA 5.0 software. The evolutionary history of isolate was inferred using neighbor-joining (N-J) method and boot strap re-samples 500 times. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site

Table 2 Comparison of the characteristic of different reported type strains of the genus *Methanospirillum* spp.

Characteristic		1	2	3	4	5
Cellular morphology		Curved rods	Curved rods	Curved rods	Curved rods	Curved rods
Motility		+	–	+	+	–
Temperature (°C)	Range	20–40	25–50	15–37	5–37	4–32
	Optimum	30	30–37	30	20–37	25
pH	Range	6.5–8.5	6.5–10.0	6.0–9.5	6.0–10.0	6.5–8.0
	Optimum	7	7.0–7.5	7.5	6.6–7.4	7
NaCl concentration (M)	Range	0.0–0.2	0.0–0.2	0.0–0.2	0.0–0.3	0.0–0.1
	Optimum	0	0	0	0	NA
Utilization of formate		–	+	+	±	–
Generation time (h)		73.43 (at 30 °C)	20.7 (at 40 °C)	32.3 (at 30 °C)	39.8 (at 30 °C)	NA
Specific growth rate (μh^{-1})		0.0094	NA	NA	NA	0.065
Source		Anaerobic digester	Sewage sludge	Soil	Sludge	Wetland Soil
Substrate utilization spectrum						

Where, Strain, 1: $T_5$3BJ (data from the present study); 2: *Methanospirillum hungatei* (Ferry et al. 1974)); 3: *Methanospirillum lacunae* (Iino et al. 2010); 4: *Methanospirillum stamsii* (Parshina et al. 2014); 5: *Methanospirillum psychrodurum* (Zhou et al. 2014); −: Negative; +: Positive; ±: very weak; NA: Not available

and phylogenetic analysis, the strain $T_5$3BJ represents *Methanospirillum* spp. and probably a novel hydrogenotrophic methanogenic species from anaerobic digester compared to available four strains of *Methanospirillum* spp. Moreover, the strain $T_5$3BJ produced CH_4 exclusively from H_2: CO_2, indicating that it could be a representative of a novel hydrogenotrophic methanogenic species of the genus *Methanospirillum*, since 3 strains of *Methanospirillum* reported earlier use both formate and H_2: CO_2 to produce methane (Iino et al. 2010). Therefore, isolation and characterization of hydrogenotrophic *Methanospirillum* from anaerobic digester may pave more interest in the methanogens from the anaerobic digesters operated with agro-industrial substrates such as PMC and NC.

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Code availability Not applicable.

Author's contribution **KALPANA A. JAIN**: Performed Research and contributed to carry out experiments; **PRADEEP C. SURYAWANSHI**: Contributed to develop methods and suggestions; **AMBALAL B. CHAUDHARI**: Designed the outline of study and critically analyzed the manuscript and data.

Data availability Not applicable.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest to disclose.

Ethics approval Not applicable.

Consent to participate Not applicable.

Consent to publish/ Not applicable.

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