

Using autochthonous *Bdellovibrio* as a predatory bacterium for reduction of Gram-negative pathogenic bacteria in urban wastewater and reuse it

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Received: May 2020, Accepted: July 2020

ABSTRACT

Background and Objectives: The microbial contamination of wastewater is associated with health risks. The aim of this study was to use the autochthonous *Bdellovibrio* potential to prey Gram-negative pathogenic bacteria as a bio-control agent to treat urban wastewater.

Materials and Methods: Thirty-six raw sewage samples were collected for isolation of *Bdellovibrio*. Double layer plaque assay was used for isolation and the isolates were identified by microscopic examination and molecular analysis. To evaluate the predatory potential for decrease number of Gram-negative pathogenic bacteria, plaque perdition assay, reduction in host cells viability by colony-forming unit (CFU) counting, reduction in optical density (OD) in co-cultures and assay of killing efficiency were carried out. Also, the raw wastewater was treated by *Bdellovibrio* then the reduction in CFU counting and reduction in OD was evaluated.

Results: Four strains of *Bdellovibrio* were isolated and were registered in Gene Bank. Clear plaques were observed after 3-6 days of incubation for all prey cells. The CFU enumerations of all preys were decreased after 48 hrs in co-cultures and raw wastewater. Also, OD was decreased down to 0.2 nm after 48 hrs.

Conclusion: These autochthonous *Bdellovibrio* strains are proposed to use for bio-control of Gram-negative pathogenic bacteria in wastewater and reuse it for irrigation in arid regions.

Keywords: Autochthonous *Bdellovibrio*; Predatory bacterium; Bio-control; Gram-negative pathogenic bacteria; Waste water treatment

INTRODUCTION

Currently, it is well known that predation is common among prokaryotes in different ecosystems. Bacteria in their natural environments are subjected to predation by bacteriophages, protists as well as predatory bacteria (1). Predatory bacteria are unique in the fact that they are cellular organisms, in con-

trast to viruses and phages, and are smaller than the prey in contrast to protists (2). Maintaining the requirement of the balance of bacteria in nature, and the ecological role of predatory bacteria due to their ability to kill prey cells, it has been suggested that they play an important role as antibacterial and bio-protection agents (3).

To date, the *Bdellovibrio* and like organisms are the most studied predatory bacteria, which are small, highly-motile and Gram-negative bacteria that prey on other Gram-negative bacteria. These predatory bacteria were discovered by chance, in 1962 by Stolp and Petzold while they were trying to isolate bacteriophages for plant pathogenic bacteria from soil. These bacteria are widely distributed in marine,

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freshwater and terrestrial ecosystems, including estuaries, seacoasts, rivers, sewage, fish pond, runoff of irrigation and man-made water supplies (4, 5). Their life cycle is biphasic, with a free-living attack phase and an intra-periplasmic growth phase that takes place in the periplasm of the bacterial prey, referred to as bdelloplasts (6, 7).

Microbiologists are interested in *Bdellovibrio* sp. because of their potential to control many pathogenic Gram-negative microorganisms as bio-control, and because of their degradative enzymes that can lyse target prey cells. Therefore, *Bdellovibrios* were mentioned as live bio-control agents to be used against Gram-negative pathogens (8).

Wastewater comes from ordinary living processes and is defined as used water from any combination of domestic, industrial, commercial or agricultural activities, surface runoff or storm water, and any sewer inflow or sewer infiltration (9). The types of microorganisms in sewage and their quantity will vary depending on the nature of sewage. Raw sewage contains millions of bacteria per ml. These bacteria include coliforms, fecal streptococci, anaerobic spore-forming bacilli, the *Proteus* group and other types arising from the human intestinal tract that may cause dysentery, cholera, and typhoid. Also, *Escherichia coli* is simultaneously a biological indicator of wastewater treatment safety an important human pathogen responsible for several diseases (10). Wastewater pollution with pathogenic microorganisms is one of the serious threats to human health, therefore, domestic and industrial wastewater without treatment, evacuates to sewers and the contaminated rainwater and another pollutant in rainwater runoff from urban areas draining to the sewers would cause a significant unfavorable impact on the water environment. Therefore, municipal wastewater treatment plants play a crucial role in reducing the microbial and pathogen load of human waste and there are many different technologies such as physicochemical and (or) microbial for wastewater treatment (11).

The goal of this study was to use the *Bdellovibrio* potential on prey Gram-negative pathogenic bacteria to decrease the number of these bacteria in sewage as a bio-control. In this regard, autochthonous *Bdellovibrio* that isolated from wastewater and registered in NCBI with the accession numbers of MH359237, was used and these isolated *Bdellovibrio* was applied for decrease number of Gram-nega-

tive pathogenic bacteria as bio-control by the plaque perdition assay, reduction in host cells viability measured by CFU enumeration, reduction of OD in co-culture and efficiency of killing assay.

MATERIALS AND METHODS

Bacterial strains and cultures. Some Gram-negative pathogenic bacteria such as *Salmonella enterica* (ATCC9270), *Klebsiella pneumoniae* (ATCC13883), *Escherichia coli* (ATCC 2592) and *Enterobacter* (ATCC 13048) were obtained from German Collection of Microorganisms and Cell Cultures GmbH (DSMZ). The overnight cultures of these bacteria were concentrated and adjusted to 1×10^9 CFU.ml⁻¹ and used separately as prey cells for the examination of the predation potential of *Bdellovibrio*. Also, the *Bdellovibrio* suspension in N-[2-Hydroxy Ethyl] Piperazin-N'-2[2-EthaneSulfonic acid] (HEPES) buffer (1×10^5 PFU.ml⁻¹) was prepared. HEPES buffer was obtained from Sigma Aldrich and all the medium cultures in this study were obtained from Quelab (12).

Sample collection. Raw sewage (36 samples) with a pH value of 7.4, BOD=55 milligrams per liter and COD=274 milligrams per liter were collected from December 2017 to March 2019 from north municipal wastewater treatment plant of the city of Isfahan (Iran) (32°44'58.7 N-51°44'02.2 E) and south municipal wastewater treatment plant of Isfahan (32°37'07.3 N-51°43'37.8 E). The raw sewage was collected with clean bottles and immediately transported to the laboratory for isolation and characterization of *Bdellovibrio* strains.

Isolation of *Bdellovibrio* strains. For isolation of *Bdellovibrio* strains, the samples were centrifuged for 5 min at 500× g, at 4°C and the pellet was discarded. The supernatant centrifuged 20 min at 20000×g at 4°C and then the supernatant was discarded and the pellet was resuspended in 4 milliliters of cold N-[2-Hydroxy Ethyl] Piperazin-N'-2[2-EthaneSulfonic acid] (HEPES) buffer, then passed through a 1.2 μm nucleopore filter, and then double layered plaque assay was performed. In brief, 10-fold serial dilutions of filtrates in HEPES buffer was prepared, then 100 μl of each dilution was mixed with 200 μl of suspension of the prey cell in 0.6% molten diluted nutrient broth agar (DNB) (1:10) dilution of nutrient broth amended

with 3 mM MgCl₂·6H₂O and 2mM CaCl₂·2H₂O. The mixture was spread over a DNB bottom agar (1.5%) and plates were incubated at 30°C for 3-6 days (12, 13).

Identification of *Bdellovibrio* strains. After 3-6 days incubation at 30°C *Bdellovibrio* plaques were cut with a sterile scalpel and resuspended in HEPES buffer, then they passed through a 0.45 µm syringe filter and were used for microscopic examination and molecular analysis to identify *Bdellovibrio* strains (12).

Electron microscopy. One drop of the plaque suspension was spotted on the microscope grid. Excess liquid was removed by blotting with filter paper and the sample was counterstained with uranyl acetate solution for 4 min and after air-dried, was examined with the transmission electron microscope (14).

Amplification of the 16S rRNA gene and DNA sequencing. Genomic DNA was extracted using the RIBO-Prep nucleic acid extraction Kit. PCR amplification of 16S rRNA was carried out using universal primer RW01 (5'-AAC TGG AGG AAG GTG GGG AT-3') and DG74 (5'-AGG AGG TGA TCC AAC CGCA-3'). Initial denaturation was carried out for 20min at 95°C. In the next 35 cycles denaturation was carried out for 1 min at 4°C, annealing at 58°C for 50 sec and extension at 72°C for 1 min. The final extension was performed for 1 min at 72°C. Products of PCR were purified and sequenced at BIO-NEER South Korea. Sequences were aligned with previously published bacterial 16S rRNA sequences in the NCBI reference databases using Blast and then the phylogenetic tree was constructed with MEGA6. *Klebsiella* was used as a prey cell to construct the phylogenetic tree, because of the high efficiency of *Bdellovibrio* killing for these bacteria (7, 15).

Predation assay. For plaque predation assay double layered plaque technique was used, as described previously, with above mentioned bacteria as prey cells. Plates were incubated for 3-6 days at 30°C and then were checked for clear plaques formation (16).

To evaluate the effect of *Bdellovibrio* on Gram-negative pathogenic bacteria two groups of samples were prepared, 200 µl of host suspension (1 × 10⁹ CFU.ml⁻¹) inoculated into the Erlenmeyer flasks contained culture medium and was used as control (Group A), and 100 µl of *Bdellovibrio* suspension (1

× 10⁵ PFU.ml⁻¹) was inoculated into the flask consists of 5 ml culture medium and 200 µl of host suspension (Group B). The flasks were incubated at 30°C at 120 rpm for 48 hrs (17, 18).

The optical density of Groups A and B at 570 nm were checked to determine the reduction of turbidity of the samples after 0, 4, 24 and 48 hrs. Also, the changes in the prey population for these two groups were measured by dilution plating and CFU enumeration after 0, 24 and 48 hrs (19-21).

Efficiency of killing assay. This assay was performed according to the Rogosky method (22). In this way, two groups of samples were prepared (as previously described) and they were incubated at 30°C 120 rpm for 4 hrs. To determine the colony-forming units at time 0 and 4 h, each group was serially diluted (10⁻¹, 10⁻², 10⁻³ and 10⁻⁴) then was plated on MacConkey Agar. The plates were incubated for 24 hrs at 37°C. The percent of Gram-negative pathogenic bacteria (used as a prey cell) were killed by *Bdellovibrio* predation was calculated using the following formula as described previously by Rogosky (22):

$$\% \text{Killed} = (1 - B_4/B_0) / (A_4/A_0) \times 100$$

Where A₀ and A₄ are the mean CFU.ml⁻¹ of Gram-negative pathogenic bacteria in the absence of *Bdellovibrio* (group A) at the time of 0 and 4 hours respectively, and B₀ and B₄ are the mean CFU.ml⁻¹ of Gram-negative pathogenic bacteria in the presence of *Bdellovibrio* (group B) at the time of 0 and 4 hrs (22).

Effect of *Bdellovibrio* on Gram-negative pathogenic bacteria in raw sewage. Two groups of samples were prepared, Group A, as control contained 250 ml raw sewage added to 500 ml flask, and group B, that contained 250 ml of raw sewage that was treated with 100 µl of *Bdellovibrio* suspension (1 × 10⁵ PFU.ml⁻¹) then both flasks were incubated at 30°C at 120 rpm for 48 hours. The OD was measured at 570 nm to determine the reduction of turbidity of the samples after 0, 4, 24 and 48 hours (12, 21).

To determine CFU of Gram-negative pathogenic bacteria in raw sewage, the samples were serially diluted and were plated on the Mac Conkey Agar medium then incubated at 37°C for 24 hrs.

Also, the variation of BOD and COD were checked in group A and B at the wastewater treatment plant of Jey Industrial Zone of Isfahan (Iran).

RESULTS

Isolation and identification of *Bdellovibrio* strains. After 3-6 days of incubation, transparent lytic plaques on the double-layer plates were observed. The size of plaques expanded upon longer incubation (Fig. 1).

The presence of such features in the lytic plaques can distinguish *Bdellovibrio* from phage plaques. The optimum temperature for *Bdellovibrio* growth is 25-30°C, and for this reason, *Bdellovibrio* isolated from the spring and winter samples did not show any lytic plaque in summer and autumn samples (2) (Table 1).

The microscopic examination revealed the small, comma shape cell with polar flagella in attack phase of *Bdellovibrio* life cycle (Fig. 2).

Stages of *Bdellovibrio* life cycle such as penetration to the prey cell, bdelloplast formation and release from the prey cell confirm the presence of *Bdellovibrio* in the tested samples (Fig. 3).

When the sequences of *Bdellovibrio* were checked in NCBI, we found 91% similarity to *Bdellovibrio* sp. HEA, *Bdellovibrio* sp. ETP, *Bdellovibrio exovorius* JSS and 92% similarity to *Bdellovibrio* sp. BDIR, *Bdellovibrio bacteriovorus* strain BRP4, *Bdellovibrio bacteriovorus* strain BEP2 and 94% similarity to uncultured *Bdellovibrio* sp. and the phylogenic tree based on 16S rRNA gene sequences was constructed (Figs. 4 and 5). These isolated strains were registered in the NCBI with the accession numbers of MH359237, MH359238, MH359239, and MH359240 respectively.

Table 1. Sampling season and plaque formation

Sampling season	Plaque formation
Spring	+ (March, April and May)
Summer	-
Autumn	-
Winter	+ (January, February and March)



Fig. 1. Expanding the size of plaques upon longer incubation a formation plaque by *Bdellovibrio* with *Pseudomonas putida* A) after 3 days, B) after 5 days, C) after 6 days

Predation assay. Clear plaques were observed in the co-culture of each prey cell with *E. coli*, *K. pneumoniae*, *S. enterica*, and *Enterobacter* after 3-6 days of incubation.

Fig. 6. shows the changes in the prey population at time 0, 24 and 48 hrs. These changes were different and were depending on the prey cell. For example, the changes for *K. pneumoniae* and *E. coli* were high.

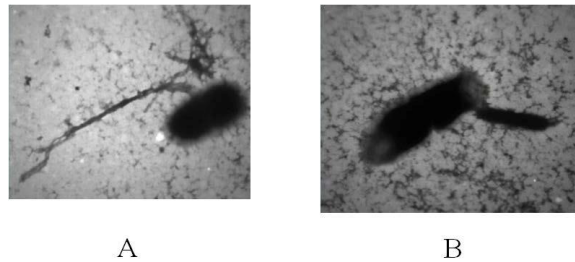


Fig. 2. Transminssion Electron Microscopy images A) a small - flagellate *Bdellovibrio*, B) a comma shape *Bdellovibrio* is attacking prey cell

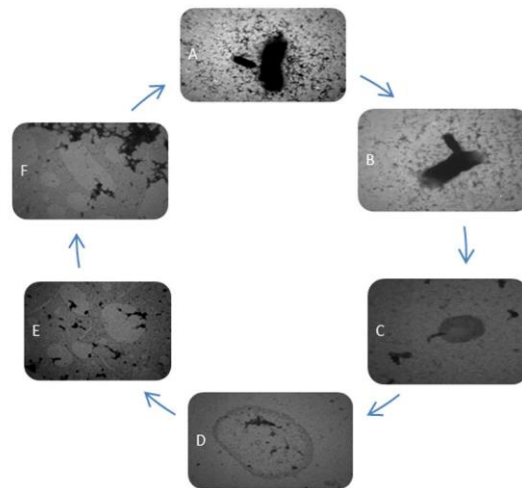


Fig. 3. The life Cycle of *Bdellovibrio*. Transminssion Electron Microscopy images showing the life cycle of *Bdellovibrio* A and B) attachment) penetration, D) Bdeloplast formation and growth, E and F) release. This life cycle lasts about 3-4 hours.

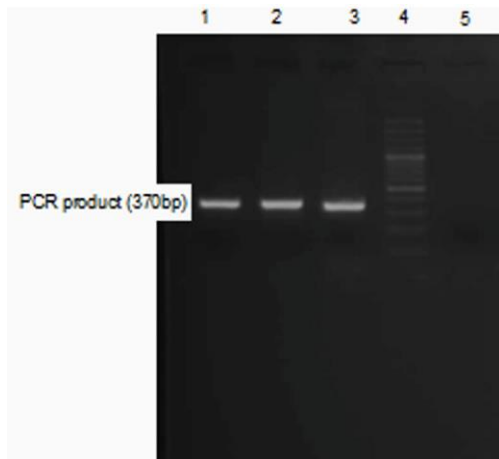


Fig. 4. PCR amplified product (370 bp) by using universal primer RW01 and DG741) MH359237, 2), MH359238, 3) MH359239; 4) PCR marker, 5) negative control

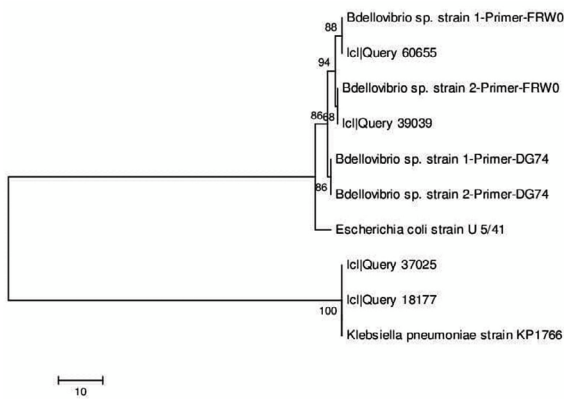


Fig. 5. The phylogenetic tree based on 16S rRNA gene sequences constructed with the neighbor-joining algorithm using the pairwise distance metric to recover their clustering pattern. One the strain that isolated in this study has 94% similarity to *Bdellovibrio* sp, therefore it was used to *Bdellovibrio* potential to prey Gram-negative pathogenic bacteria as a bio-control agent to treat urban wastewater. Bootstrap value (~50%) based on 1000 replicates are shown at branch nodes.

The changes of OD in co-culture and free *Bdellovibrio* culture were measured at 0, 4, 24 and 48 hrs. These results showed that the OD in co-culture was decreased, because the *Bdellovibrio* preyed the host cells (Fig. 7). All experiments were done in triplicate.

It is worth mentioning that the OD in co-culture was increased after 48 hrs and the reason for this decrease and increase of OD refers to the *Bdellovibrio* life cycle. When the nutrient supply from the prey

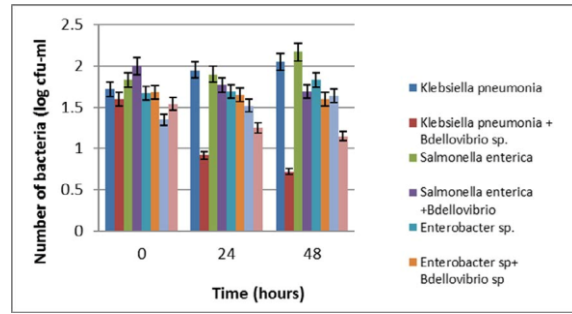


Fig. 6. The effect of isolated *Bdellovibrio* on prey population at time 0, 24 and 48 hours
All experiments were done in triplicate.

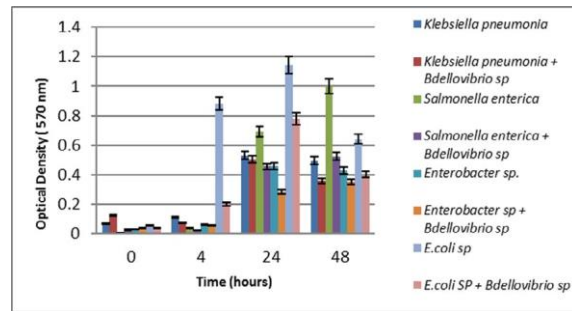


Fig. 7. The changes of OD in co-culture and free *Bdellovibrio* culture at time 0, 4, 24 and 48 hours

cytoplasm has been exhausted by *Bdellovibrio*, the filament fragments into unit size cell and after fragmentation, the cells become flagellate, then synthesize a lytic enzyme to lyse prey cell, realize the flagellate attack phase cells and the life cycle of *Bdellovibrio* restarts. In this time the OD of co-culture increase (2).

The efficiency of killing assay. The efficiency of *Bdellovibrio* killing for each prey cell was also measured by using the Rogosky method (2011). The efficiency of *Bdellovibrio* killing was 92% for *E. coli*, 99% for *Klebsiella*, 72% for *Enterobacter* and 45% for *Salmonella*.

Effect of *Bdellovibrio* on Gram-negative pathogenic bacteria in raw sewage. The number of Gram-negative pathogenic bacteria were decreased in co-culture and in wastewater treated by *Bdellovibrio* the OD was decreased too (Fig. 8A) these results show that *Bdellovibrio* can alleviate the microbial contamination in wastewater therefore, it is possible to reuse sewage in some cases such as industrial or

irrigation uses.

As shown in Fig. 8B, BOD5 in co-culture was increased. However, COD did not change at all samples and it was measure for 5 days. According to the rule of thumb of Iran sewage standard (st-04.00), the value of COD should be less than 100 mg L⁻¹, but this value was decreased to 72 mg L⁻¹ that is within the acceptable range.

DISCUSSION

Two of the biggest challenges that mankind is facing today are water pollution and water scarcity, that due to some factors such as increasing population, climate changes, industrial development and increasing water use per capita. Because of the scarcity of water resources, it is important to introduce new ways for water supply that would be easily accessible, cheap and applicable to different areas (23, 24).

In this way, the need for wastewater reuse has been identified by several global and internationally recognized organizations. The reuse of wastewater will become an important part of the water supply for various water sectors, especially irrigated agriculture (25, 26). For example, farmers are compelled to use wastewater for irrigation due to a shortage of food quality especially in the urban area. Therefore, the management of irrigation water requires serious attention (27). In this way, there are also broad technologies available including sequencing batch reactor, membrane filter and ultraviolet disinfection for decrease or eliminate human health and environmental risks, but these manners are the coast and require special facilities (28).

Hereupon, some scholars believe that bacteriophages and predatory bacteria, as natural biological systems hold promising bio-control potential for control of pathogenic bacteria. Periasmay and Sundaran (2013) used specific phages to the reduction of patho-

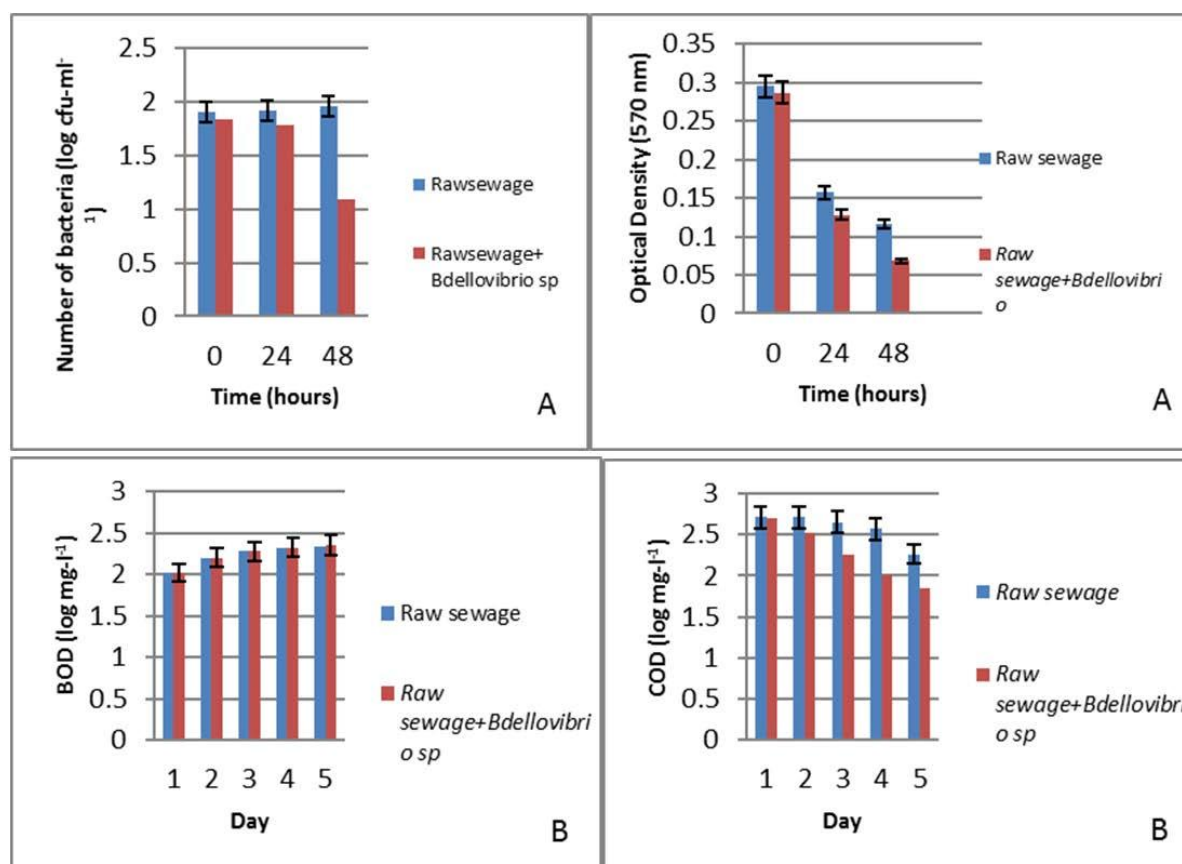


Fig. 8. The effect of isolated *Bdellovibrio* on raw sewage, A showed that in co-culture the number of Gram-negative bacteria and OD decreased, therefore *Bdellovibrio* used the Gram-negative bacteria in wastewater as prey cell. B showed the effect of isolated *Bdellovibrio* on BOD and COD in co-culture and showed that *Bdellovibrio* can change these items to accepted rate. All experiments were done in triplicate.

gens from hospital wastewater (29), but bacteriophage have high specificity for target bacteria and this object limits their application for wastewater treatment, while predatory bacteria such as *Bdellovibrio* have a wide prey range among Gram-negative bacteria (13).

On the other hand, extensive use of antibiotics as humans and veterinary medicines has increased; therefore, wastewater contains antibiotic-resistant bacteria, which pose a threat to human health. If *Bdellovibrio* were used in the biological control of pathogens, although there is likely to be some resistance to predation, it is also likely that the number of resistant bacteria will be low. According to this subject, *Bdellovibrio* would be an effective biological control or therapeutic agent (3).

Although these organisms are used to treat the unwanted pathogens in the aquatic and industrial setting, the safety of *Bdellovibrio* in humans should be considered. Ibba et al. (2013) reported the presence of *Bdellovibrio* in the gut of the healthy individuals that can be the strongest evidence to support the safety of *Bdellovibrio* in humans (30). Shanks et al. (2014) found that *Bdellovibrio* in its lipopolysaccharide structure had a unique lipidA portion that was less immunogenic than typical lipopolysaccharide (5). According to this data, there are several benefits to using *Bdellovibrio* as a predatory bacterium for wastewater treatment and reuse it: (1) it is easy and inexpensive to access because *Bdellovibrio* isolated from raw sewage (2) since the *Bdellovibrio* is available in wastewater, this manner is applicable to different areas (3) there is no human and environmental risk in using this manner.

In this way, Richards et al. showed that predatory bacteria as a natural modulator of *Vibrio parahaemolyticus* and *Vibrio vulnificus* in seawater and oysters (14). El-Shanshoury et al. (2016) isolated *Bdellovibrio* sp. from wastewater and used their potential application in the control of *Salmonella paratyphi* in water (8). Also, Oyedara et al. (2016) isolated *Bdellovibrio* sp. from the soil samples in Mexico and used their potential application in control of pathogens (12).

About the reuse of wastewater, membrane bioreactor (MBR) and biological aerated filter (BAF) were used by Ren et al. (2019) to treat gray water, but the main drawback of MBR technology in comparison to conventional systems is membrane fouling caused by microbial attachment to the membrane

surface (26). Yu et al. (2017) used quorum quenching isolate of *Bordetella hinzii* S3 to prevent fouling in MBR systems, but using predatory bacteria such as *Bdellovibrio* can be used for cleaning of MBR system (31). In this way, Yilmaz et al. (2014) used the *Bdellovibrio bacteriovorus* as a biological cleaning method for the MBR system (9).

CONCLUSION

Based on the discussion of the National Science Information Center and the results published in the scholar journals, it seems there are not enough studies about the isolation of autochthonous *Bdellovibrio* in Iran. In this report, *Bdellovibrio* was isolated from sewage as an autochthonous strain to be used as a bio-control agent. The effect of autochthonous *Bdellovibrio* on Gram-negative bacteria as a prey cell was examined and was shown that autochthonous *Bdellovibrio* can prey Gram-negative pathogenic bacteria as prey cell and decrease the number of these bacteria in wastewater. Also, high efficiency of *Bdellovibrio* killing of *Klebsiella* and *Escherichia coli* as important Gram-negative pathogenic bacteria, that causes important disease in human, was shown. On the other hand, the changes BOD and COD in wastewater that treated by *Bdellovibrio* shown that this predatory bacteria can make wastewater suitable for reuse. Based on these results, the authors propose that it is possible to utilize the autochthonous *Bdellovibrio* to bio-control Gram-negative pathogenic bacteria in wastewater and reuse it for agriculture and plant irrigation.

ACKNOWLEDGEMENTS

This article was extracted from Ph.D. thesis (Thesis code is 15730507951010) and the authors thank the Microbiology laboratory staff of Islamic Azad University of North Tehran branch and Islamic Azad University of Falavarjan Branch.

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