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ORIGINAL ARTICLE

Suppression of predominant interfering bacteria in the purification process of myxobacteria

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ABSTRACT

Background and Objectives: Myxobacteria initially recognized by their complex life cycle and social behavior are progressively explored for their bioactive secondary metabolites. However, isolation of myxobacteria usually is accompanied by bacterial and fungal contaminations due to the direct cultivation of soil on isolation media, which results in severe challenges in the purification of myxobacteria. Therefore, it is necessary to improve their purification techniques from natural samples to the discovery of new biomolecules.

Materials and Methods: In the present study, six physichochemical methods were assessed for their efficacy in the purification of myxobacterial strains and specially from contaminants of *Microvirga* spp.

Results: Among the evaluated treatments, purification of fruiting bodies using a combination of ultrasonication and heat treatment was identified as the effective protocol with 80% success rate in the purification of myxobacterial strains and reducing up to 90% of the contaminating bacteria.

Conclusion: Concerning the problematic contamination of myxobacterial isolates, the introduced approach can retrieve the myxobacterial strains which are often suppressed by the over growth of contaminations especially root symbiotic bacteria namely *Microvirga* spp.

Keywords: Myxobacteria; Strain purification; Heat treatment; Ultrasonication

INTRODUCTION

Myxobacteria are Gram-negative bacteria with distinctive social behavior, metabolic and morphological differentiation which leads to distinct cell types of vegetative cells and myxospores (1). Under starvation conditions, they are move by gliding on solid surfaces and form fruiting bodies consisting of resistant forms of cells called myxospores (2, 3). The large genome size of approximately 9-13 Mb is among characteristics of myxobacteria and they are valuable resources for isolation of the new bioactive molecules with the genomic capacity in secondary metabolite production which even contend the well-known resources of Actinomycetes, fungi and *Bacillus* spp. (4). Myxobacteria can produce diverse secondary metabolites with desirable activities, including anti-viral, anti-fungal, anti-cancer, anti-bacterial, and a variety of hydrolytic enzymes. The diversity and unique structural properties of these

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secondary metabolites are what make these social microbes highly attractive for industry. The meta genomic analysis has indicates that there are many new genera and species especially from suborders *Sorangiineae* and *Nannocystineae* still not cultured and unknown (4, 5). As myxobacteria mostly are isolated by direct transferring of soil samples into the isolation media, fungal and bacterial contaminations often are present during the purification process. The distinct difference between isolation and purification is their end product, which is a retrieval of the strain from its natural niche and separation from other microorganisms as a pure culture, respectively. Therefore, the removal of the accompanying cells and transforming them into a pure state of the culture is the major rate-limiting factor in working on members of this order of bacteria (6). Besides, myxobacteria move on solid media due to the slime production and therefore they can convey contaminates with themselves including other bacteria, fungi, and amoebae (7).

The conventional method of baiting is often implemented in the eradication of bacterial contamination in myxobacteria culture and there are possibilities for inhibiting the fungal growth by adding cycloheximide or nystatin to the isolation media (8). The bacterial contaminations of the myxobacterial cultures are often more resistant to elimination than the fungal contaminants and novel methods for purification of myxobacteria must be introduced to alleviate this rate-limiting stage. Since the last decades of the twentieth century, a great effort has been made to illustrate the details of simple and efficient strain purification techniques, which can lead to the identification of new myxobacterial taxa (9).

Symbiotic bacteria in soil or plant roots such as *Microvirga* have similar growth, morphological and cultural characteristics to myxobacteria including compact and circular colonies, with 2 mm in diameter resembling the sporangioles of myxobacteria, and interfere in the isolation process of myxobacteria (10). The genus *Microvirga* belongs to the phylum *Proteobacteria*, order *Rhizobiales* and family of *Methylobacteriaceae* with 17 described species from 2003 until to date (11). Therefore, the nomination of the appropriate method for the exclusion of such contaminations can accelerate the purification of myxobacteria. The strains of *Microvirga* also have similar oxygen and nutrient demand to mycobacteria. The most favorable conditions for the growth of most of the well-known strains of this group are neutral pH and high concentrations of minerals, respectively. Most cultivable strains of *Microvirga* grow within a temperature range of 25 to 30°C and optimal pH 7.0-8.5 which is similar to the conditions favored by myxobacteria as well. They are metabolically catalase-positive and oxidase-negative, and such as most of the cellulolytic myxobacteria use nitrate as a nitrogen source. Yeast extract is the best source of carbon for these bacteria. In addition, with the presence of a polar flagellum, *Microvirga* has a high rate of growth and movement in the culture medium than myxobacteria, thus, in the culture medium, the isolation of myxobacteria contaminated with these bacteria will be challenging (12, 13). In this study, the development of a simple method for the purification of myxobacterial cells is introduced. The efficacy of the purification methods including ultrasonication, heat treatment, a combination of ultrasonication and heat treatment, using antibiotics, crystal violet, and filter paper for the elimination of the contaminations of myxobacterial cultures were assessed in this study.

MATERIALS AND METHODS

Chemicals and microorganisms. The chitin and corn powder used in these experiments were prepared from shrimp shells and corn seeds. The corns and shrimp shells were purchased from local market and washed thoroughly using tap water and finely grinding after drying. The antibiotics used in cultivation were purchased from Sigma-Aldrich (USA), inorganic salts and vitamins were purchased from Merck (Germany). The microorganism used for baiting of myxobacteria on isolation media was *Escherichia coli* ATCC 8739 (UTMC 1407).

Isolation of myxobacterial strains. Myxobacteria were isolated from 30 soil samples and 10 bark samples which collected from different areas in Iran. The selection of the habitats was based on the recognition of myxobacteria as indigenous inhabitants of the soil, on dung pellets, on decayed wood and plant material (14). All the samples were air-dried for 3-4 weeks before storage in vials at room temperature in darkness, to prevent the growth of unwanted contamination microorganisms during the isolation process. The isolation of myxobacteria was carried out based on the standard procedure of bacterial baiting tech-

nique using dead cells of *E. coli* on WAT agar, chitin agar and corn agar media (Table 1). The culture solid media were incubated under humid conditions (40-60%) at 28ºC for up to three weeks. The myxobacterial cells were identified by the detection of fruiting bodies or swarm on agar under the stereomicroscope (Motic, China) (15).

Purification techniques of myxobacteria from their accompanying microorganisms. In this study, six methods were evaluated in the purification of myxobacteria consisting of ultrasonication, heat treatment, the combination of ultrasonication and heat treatment, antibiotic treatment augmentation of WAT medium ($CaCl_2.2H_2O$ 0.1%; agar 1.2%; pH 7.8, after autoclaving, 50 µg/mL-1 cycloheximide was added to the medium) by 0.05 % (w/v) crystal violet and maintenance of fruiting bodies in dry condition.

Purification of myxospores by ultrasonication. For the ultrasonication treatment, fruiting bodies of myxobacteria were removed from the contaminated cultures and then were dissolved in 1 mL phosphate buffer pH7.0. Subsequently, samples were sonicated for 1-3 min with frequencies of 37 kHz and 80 kHz.

Finally, myxospores suspension were cultured on VY/2 agar (Baker's yeast 5 g/L, $CaCl_{2.}$ $2H_2O$ 1 g/L, agar15 g/L, Vitamin B_{12}0.0005g/L) and CY medium (Casitone 3 g/L, yeast extract 1 g/L, $CaCl_{2.}$ $2H_2O$ 1 g/L, and agar 15 g/L) and were incubated at 28ºC for 3-10days (16).

Purification of fruiting bodies using heat treatment. The fruiting bodies of myxobacteria were removed from the contaminated cultures and were inoculated in 1 mL phosphate buffer pH 7.0. Then, the contaminated fruiting bodies were incubated at a range of temperatures (30, 40, 50 and 60°C) for 2-5 min and then the suspension was cultured on VY/2 plates and WAT agar at 28ºC for 3-10 days.

Combination of ultrasonication and heat treatment. In the combination treatment of ultrasonication and heat treatment, samples were inoculated in phosphate buffer and then incubated at 30, 40, 50 and 60°C for 2-5 min and subsequently were sonicated for 1-3 min with frequencies of 37 kHz and 80 kHz. Finally, either one of the suspensions was cultured on VY/2 agar and CY/2 medium and the fruiting bodies in suspension were transferred to VY/2 and WAT agar by sterile needle and was incubated at 28ºC for 3-10 days.

Purification of myxobacteria using antibiotic treatment. Since myxospores do not germinate in nutrient-rich media in presence of fast growing heterotrophic bacteria, they can tolerate to the exposure of the toxicants such as antibiotics. The contaminants can be suppressed by the inhibitors of cell fission and the fruiting bodies are transferred into a suitable growth medium on which their myxospores can germinate without being affected by the fast growing contaminant cells (17).

Contaminated fruiting bodies were then transferred by a sterile needle to WAT agar plates containing ampicillin 10 µg.mL^{-1} and ciprofloxacin 5 µg.mL^{-1} or containing tetracycline 8 µg.mL^{-1} and imipenem 1 µg.mL^{-1} or containing ampicillin 50 µg.mL^{-1} or ciprofloxacin 25 µg.mL^{-1} (Table 2). Then, the plates were incubated at 28ºC for 3-10 days (18). The criteria for selecting the antibiotics was based on the antibiotic resistance pattern of myxobacterial genera and antibi-

Table 1. Innovative isolation media (Chitin agar, Corn agar and WAT agar) introduced in this study for recovery of myxobacteria 3(g/l) modified from base medium WAT agar (36, 37).

Media	Chitin agar		Corn agar		WAT agar	
Ingredients	Chitin powder	5 g	Corn powder	5 g	-	-
	$CaCl_2 \cdot 2H_2O$	1 g	$CaCl_2 . 2H_2O$	1 g	$CaCl_2 . 2H_2O$	1g
	$MgSO_4 . 2H_2O$	1g	$MgSO_4 . 2H_2O$	1g	$MgSO_4 . 2H_2O$	1 g
	KNO_3	1g	KNO_3	1g	KNO_3	1 g
	$(NH_4)_2SO_4$	0.2 g	$(NH_4)_2SO_4$	0.2 g	$(NH_4)_2SO_4$	0.2 g
	K_2HPO_4	0.2 g	K_2HPO_4	0.2 g	K_2HPO_4	0.2 g
	$MnSO_4 \cdot 7H_2O$	0.1 g	$MnSO_4 . 7H_2O$	0.1 g	$MnSO_4 \cdot 7H_2O$	0.1 g
	Agar	12 g	Agar	12 g	Agar	12 g
Final pH	7.5		7.7		7.5	

Table 2. Types of antibiotics applied as the purification treatment of myxobacteria.

Antibiotic Regimes	Concentration (µg. mL^{-1})
Ampicillin	50
Cephalexin	25
Tetracyclin+Imipenem	8 + 1
Ampicillin+ Cephalexin	10 + 5

otic susceptibility profile of *Microvirga* spp. (19).

Augmentation of WAT medium by 0.05% (w/v) crystal violet. Another purification technique was the growth of fruiting bodies in WAT medium modified by the addition of 0.05% (w/v) crystal violet (20).

Purification of fruiting bodies by air-drying. In the method of purifying the myxobacterial fruiting bodies in dry conditions, sterile filter paper was placed in the sterile plates. The fruiting bodies were transferred by sterile needle onto a filter paper in an empty Petri dish and the plates were loaded into a desiccator for four weeks, then paper filter containing the fruiting bodies was cultured on VY/2 agar medium. The plates were incubated at 28ºC for 3-10 days (20).

Verification of the purification process. The purity of the myxobacteria was ensured through the assessment of the growth of contamination in CY liquid medium and the microscopic observation of the probable contaminant cells following Gram staining (21). The suspensions were cultured on VY/2 agar and the plates were incubated at 28ºC for 14 days to verify the eradication of the other heterotrophic contaminations.

RESULTS

Isolation of myxobacteria. A total of five myxobacterial strains were isolated from 30 soil samples and 10 bark samples collected from different areas in Iran. According to the morphological traits (shapes of vegetative cells, myxospores, fruiting bodies and swarm pattern) and molecular characterization (nucleotide sequence of 16SrRNA gene), the isolates were classified into four genera, including *Myxococcus, Corallococcus, Archangium* and *Cystobacter*. The isolates were associated with bacterial contamination with *Microvirga* spp. and were subjected to physical and chemical treatments for elimination of the contaminating strains.

Efficacy of the applied purification treatments. The results of the ultrasonic treatment showed that myxospores survived during the first two minutes of exposure to ultrasonic frequency of 37 and 80 Hz. The elevated frequency that harbors the increased energy transfer would even eliminate myxospores. In this method, only one out of five myxobacterial strains survived and 30% of the *Microvirga* load was removed. Therefore, in the method of eliminating contamination by sonication, the intensity of the applied frequency was the determinant element in the removal of contamination and preservation of myxospores.

Moreover, purification of fruiting bodies of myxobacteria by heat treatment showed that heating the fruiting bodies in a phosphate buffer (pH7.0) for 2-5 min at 50ºC or 60°C eliminated the contaminants, while the myxospores within the fruiting bodies survived in the treatment. In this treatment, the survival rate of myxobacteria isolates and the elimination rate of *Microvirga* were 40% and 20%, respectively.

Another applied purification approach was performed by a combination of ultrasonic and heat treatments. In this method, first, part of the contamination was removed from fruiting bodies by heating them for 2-5 min at 50ºC or 60°C and then were treated by ultrasonic exposure at frequencies of 37KHz (for 3 min) or 80 KHz (for 2 min). In the combined approach, all contaminants were inactivated; especially root symbiotic bacteria namely *Microvirga* spp. which are among the most challenging type of contamination in the purification of myxobacteria. Thus, this method led to an 80% survival of myxobacteria (including *Cystobacter sp*., *Myxococcus* sp., *Corallococcus* sp. and *Archangium* sp.) and a rate of 90% removal of the *Microvirga* strains.

In some cases, where bacterial contaminants are hard to eliminate, antibiotic cocktails are frequently used as the last option to purify the strains (22).

The results of the purification treatment using antibiotics showed that a mixture of two antibiotics (containing 10 µg.mL^{-1} of ampicillin and 5 µg. mL^{-1} of ciprofloxacin or containing 8 µg. mL^{-1} of tetracycline and 1 µg. mL^{-1} of imipenem) led to the removal of contaminants. The synergism effect of antibiotics was observed in the combinatorial antibiotic regimes. The lower concentration level of each antibiotic, led to an additive effect while a higher amount of each did not provide the same efficacy in the pure form of antibi-

otics. The survival rate of the strains of two genera including *Myxococcus*, and *Corallococcus* was up to 40% by this method and the same percentage of suppression was observed for *Microvirga* spp.

The method of WAT medium modified by the addition of 0.05% (w/v) crystal violet for purification of myxobacteria is suitable for the inhibition of Gram-positive bacteria from the natural substances. In this method, one of the five species called *Cystobacter* sp. was survived (which represents a 20% surviving rate). Moreover, only a 10% elimination rate has been achieved in the case of the members of the *Microvirga* genus.

The maintenance technique of fruiting bodies on the paper filter which is known as a suitable approach for the preservation of myxobacteria, up to 70% of the contamination was eliminated through this method in this study. By applying this method, three strains including *Cystobacter* sp. UTMC 4501, *Myxococcus* sp. UTMC4502 and *Archangium* sp. UTMC4504, were survived representing a 60% surviving rate and 70% removal rate for *Microvirga* strains was attained Figs. 1 and 2.

In conclusion, using a combination of heat-ultrasonic treatment led to 80% survival rate of myxobacterial strains and 90% elimination rate for *Microvirga* spp. While only less than half of the myxobacterial strains (40%) grew purely following the implementation of sole heat or antibiotic treatments. Moreover, the sole application of ultrasonic treatment and growth in the presence of crystal violet showed the least recovery rate limited to 20%. Furthermore, air-drying of fruiting bodies on the paper filter for four weeks showed the purification of over half of the myxobacterial strains (60%). Therefore, purification of fruiting bodies by the combination of ultrasonication and heat treatment was identified as an efficient technique to eliminate the accompanying strains especially the dominant contaminant of *Microvirga* (Figs. 1 and 2).

DISCUSSION

Myxobacteria are an emerging microbial source for biotechnological exploitation, due to a rich production spectrum of bioactive compounds. The myxobacterial cells biosynthesize a diversity of extracellular molecules, which are of considerable potential industrial value due to their unique structural properties, such as antibiotics, carotenoids, exopolysaccharides, polyunsaturated fatty acids and a variety of hydrolytic enzymes (5). This has made these social bacteria highly attractive for drug discovery and has been one of the highlighted source to develop bioactive compounds in the future (23). Therefore, the isolation and purification of unexplored groups of myxobacteria is of great value in discovery of new taxa and prospecting their biosynthetic potential as they can produce high levels of diverse compounds mainly in pure culture. In contrary to the necessity, only a few methods for purification of myxobacteria have been presented so far such as transferring

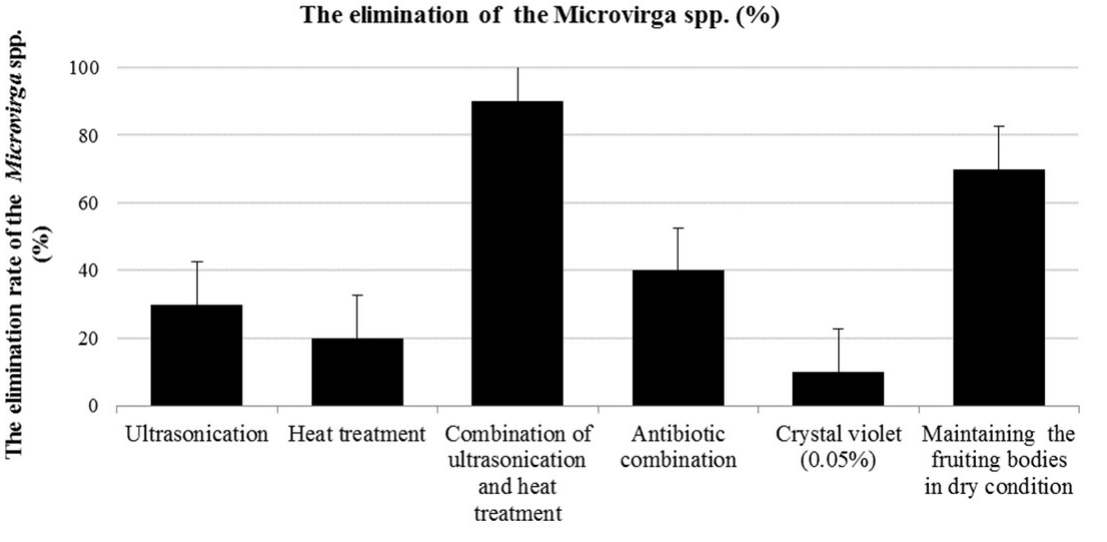

Fig. 1. The efficiency of the purification methods in elimination of the *Microvirga* spp. as the main interfering contaminant of growth media used in isolation of myxobacteria.

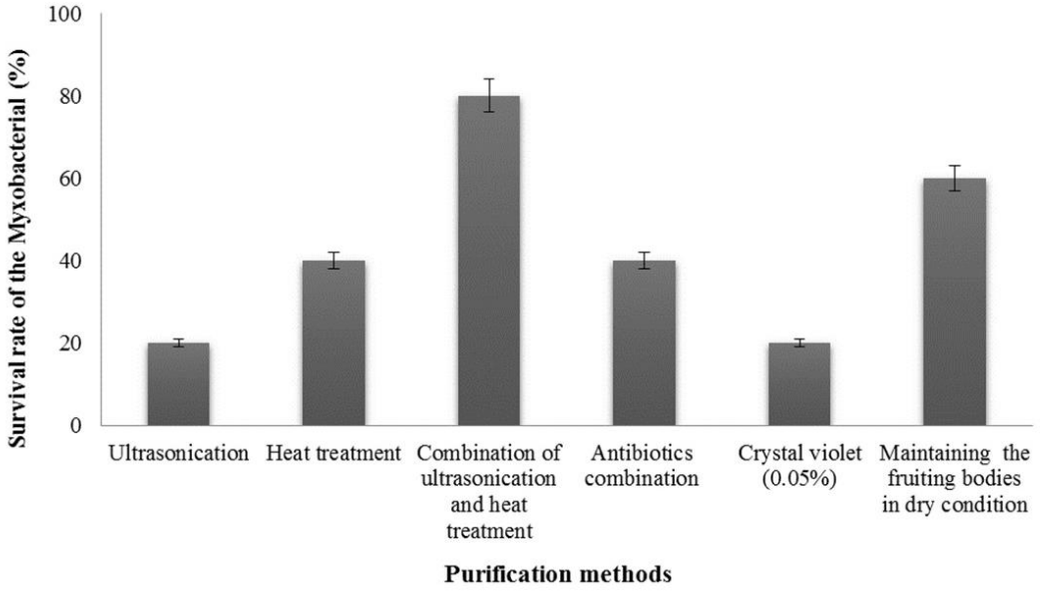

Fig. 2. The survival rate of the myxobacterial strains in response to the treatments used for suppression of accompanying contaminants.

the fruiting bodies using sterile fine syringe tips (4) which is not effective enough as the adhering contaminants still remain on the surface of the fruiting bodies. The purification and selective isolation of a variety of bacterial taxa have been reported using various methods comprising physical treatments and application of chemical agents. We adapted some of these methods to amendments and applied them with the aim of selective purification of myxobacteria. In previous studies, a wide range of physical treatments, such as ultraviolet radiation (24), ultrasonic waves and super high frequency (SHF) radiation (25, 26) have been introduced for selective isolation and purification of some group of bacteria. Among the published methods, purification of myxospores using ultrasonication has also been reported (27). Following a related study, Jiang et al. reported that ultrasonic treatment of soil samples for 40 s increased the isolation rate of rare actinomycetes, while it reduced the cell number of other types of bacteria (28). The obtained results in the present study including the survival of myxospores and the reduction of contamination by an ultrasonic exposure was in accordance with the former reports and suggesting the ultrasonic treatment as the fastest procedure to purify the myxobacteria.

According to a previous report on the purification of myxospores by heat treatment (17), it was shown that myxospores in distilled water often resist the heat treatment up to 58°C, while the majority of the contaminant does not. The intensity of frequency and duration of the exposure as the cardinal factors in the elimination of contamination and maintenance of myxospores, were determined for the myxospore of four myxobacterial genera in the current study. The heat treatment of air-dried soil samples at 100 or 120°C is recognized as pre-treatment for the reduction of vegetative cells in the isolation samples to support the retrieval of sporulating cells (29). Therefore, the thermal treatment has been shown effective in the selective isolation of some other spore producing genera such as *Micromonospora* spp. and *Streptosporium* spp. (30) showing that thermal treatment facilitated the selective separation by effective elimination of the non-heat resistant cells. In the present study, elimination of the *microvirga* spp. and survival of myxospores within the fruiting bodies during thermal treatment emphasizes the effectiveness of this universal treatment in the selective purification of myxobacteria. Nevertheless, the contamination of slime producing genera of *Cystobacter* and *Archangium* can only be removed by the combined treatments of ultrasonication and heat. The presence of the slime layer makes the purification transience and with a higher rate of failure as the transferred fruiting bodies on a new culture medium often contain associated cells of contaminants adhering to the slim layer. Therefore, the purification process of these

genera by using the conventional method is often prone to fail and can be substantially improved by the application of introduced combined treatment of ultrasonication and heat.

In this study, the combined ultrasonication and heat treatment was applied as a combinatorial purification strategy and revealed enough efficiency for the various types of contaminants; especially root symbiotic bacteria namely *Microvirga*, which is among the dominant impurities in the culture of myxobacteria.

Purification protocol with the aid of antibiotic treatments can also be useful provided that the mature myxospores are present (31). By this approach, the isolation of *Myxococcus* from soil could be accelerated by plating the soil samples directly on a medium containing antibiotics which inhibit the growth of non-sporulating cells (32). The suppression of bacteria exempting the myxobacterial cells is challenging as the most antibacterial antibiotics also suppress the myxobacterial growth. A mixture of two antibiotic regimes including ampicillin 10µg. mL^{-1} and ciprofloxacin 5 µg.ml^{-1} versus tetracycline 8 µg.ml^{-1} and imipenem 1 µg.ml^{-1} exhibited synergistic effect in contaminant removal compared to the single antibiotic treatments even at higher concentration. According to the antibiotic susceptibility spectrum of the myxobacterial genera described in the literature, it can be concluded that most of the genera are resistant to low concentration (10-20 µg. mL^{-1}) of ampicillin, ciprofloxacin and tetracycline (16). In addition, the selection of the antibiotics in this study was based on the antibiotic susceptibility and resistance of genus *Microvirga*; with sensitivity to penicillin 30, imipenem 20 ,ampicillin 40, neomycin 10, tetracycline 30, streptomycin 40, ciprofloxacin 20, chloramphenicol 10, spectinomycin 100 µg. mL^{-1} and resistant to vancomycin 100, aztreonam 30, erythromycin 15, kanamycin sulphate 30, nitrofurantoin 40 and gentamicin 30 µg. mL^{-1} (19, 33). Although most of the myxobacterial genera are susceptible to imipenem, its presence at less than 10 µg. mL^{-1} concentration, along with other antibiotics in the culture media, eliminates the contamination and maintains the myxobacterial survival at an acceptable rate.

Distinct types of chemical compounds such as tween 80 (32, 34), chlorhexidine gluconate and antibiotics (30) have been used in selective isolation of bacteria from the soil samples. In a study conducted to evaluate the efficacy of crystal violet in the purification of myxobacteria, 36 myxobacterial strains out of 85 isolated strains could be purified using the crystal violet (0.1%) which was equivalent to 42% of survival rate (20). In accordance, the results of the present study show that using crystal violet in the myxobacterial culture media could be moderately beneficial for the removal of contaminating strains. In this study, the amount of crystal violet used in WAT medium to remove the contaminants in a purification approach was modified based on a previous report (20), and its optimal amount was obtained empirically 0.05% (w/v). The antibacterial action of crystal violet is attributed to the formation of the unionized complex of the cell constituents with the dye and the toxic effects of crystal violet can be diminished by reducing its concentration and increasing the pH of the environment. Hence, this dye has an inhibitory effect on interfering bacteria in the purification process of myxobacteria at a very low concentration while the growth of the fruiting bodies was suppressed using 1% (w/v) of crystal violet. Additionally, by considering the fact that crystal violet confines the growth of Gram-positive bacteria, including this compound in the growth medium could reduce their co-cultivation with myxobacteria.

As a preservation method, pieces of agar that contain fruiting bodies can be placed on sterile filter paper in the petri dish and get dried in an evacuated desiccator and stored for years (35). Although this method has not been frequently used for the maintenance of myxobacteria, most of the contaminants in this study were eliminated by this approach and can be suggested as an effective method in the purification of myxobacteria in addition to its original usage for their preservation purposes. Nevertheless, the efficiency of the purification was genera dependent and for instance, the genus *Myxococcus* with any type of contamination often could be purified by transferring its fruiting bodies with a sterile needle onto another culture medium. However, members from genera such as *Cystobacter* and *Archangium*, due to the production of a layer of slim are not readily purified by this approach.

CONCLUSION

In conclusion, considering the diverse characteristics of myxobacterial genera, each of the purification methods evaluated in this study showed efficiency only for a fraction of myxobacterial genera. Howev-

er, the combination of ultrasonication and heat treatment was recognized as a universal treatment for the best attainable purification result, which covers the majority of myxobacterial genera. The combination of ultrasonication and heat regimes can especially be effective in the purification of myxobacteria from forest and plant rhizosphericarea, which usually has a considerable population of myxobacteria as well as the *Microvirga* spp.

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