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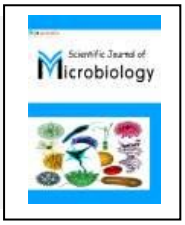

Original article

Antimicrobial activity bioactive compounds produced by *Exiguobacterium acetylicum* PTCC1756 against pathogenic bacteria

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ABSTRACT

Almost all forms of life in the marine environment (bacteria, algae, fungi, etc…) have been investigated for their natural product content. In the last several decades, plants, animals and microbes from the marine environment have revealed a portion of what is clearly a tremendous source of structurally diverse and bioactive secondary metabolites. *Exiguobacterium acetylicum* is a Gram positive, rhizospheric, yellow pigmented bacteria isolated from an apple orchard rhizospheric soil, facultative aerobic, motile with peritrichous flagella and their growth ranges are from _2.5 to 40 $^{\circ}C$. These micro organisms In separate invitro assays it was found to inhibit the growth and development of *Rhizoctonia solani*, *Sclerotium rolfsii*, Pythium and *Fusarium oxysporum*. The volatile compound produced by the bacterium was found to be the most potent in inhibiting the hyphal fungus and pathogens bacteria. Present study aims to isolated and detect the surfactants produced by *Exiguobacterium acetylicum* PTCC 1756 For this purpose, a suspension equal to 0.5 McFarland was prepared from the fresh growth of following standard clinic strains, *Escherichia coli* PTCC 1533, *Shigella Flexneri* PTCC 1234, *Staphylococcus aureous* PTCC 1112, *Salmonella enterica* subsp. *enterica* serovar *Paratyphi* B PTCC 1231 and the plated on the Muller Hinton agar. Supernatant of *E. acetylicum* growth was prepared in 15000 ppm for 30 minutes and was utilized. To study on the antimicrobial activity of *E. acetylicum*

PTCC1756, Muller- Hinton agar was used. For this purpose, Disk Diffusion Agar and Well Diffusion Agar techniques were used to define MIC. These micro organisms growth supernatant displayed a good antimicrobial property and the maximum effect was observed during the well diffusion technique against the standard strain *Salmonella enterica* subsp *enterica* serovar *paratyphi* PTCC 1231 with 8.5 mm no growth halo.

1. Introduction

Exiguobacterium was first described in 1983 by Collins et al. In 1994, Farrow et al. included the species formerly identified as *Brevibacterium acetylicum* incertae sedis into the genus *Exiguobacterium*, as *E. acetylicum* (Farrow et al., 1994). 11 new species have been added to the genus (Chaturvedi et al., 2008; Chaturvedi and Shivaji 2006; Crapart et al. 2007; Fruhling et al. 2002; Kim et al. 2005; Lopez-Cortes et al. 2006; Rodrigues et al. 2006; Yumoto et al., 2004). *Exiguobacterium* spp. These micro organisms have been detected in Siberian permafrost, temperate and tropical soils, have been isolated from, or molecularly detected in, a wide range of habitats including cold and hot environments with temperature range from -12 to 55 $^{\circ}C$. The *Exiguobacterium* genus comprises psychrotrophic, mesophilic, and moderate thermophilic species and strains (Vishnivetskaya et al., 2005), with pronounced morphological diversity (ovoid, rods, double rods, and chains) depending on species, strain, and environmental conditions (Vishnivetskaya et al., 2007). *Exiguobacterium acetylicum* is a rhizospheric, Gram positive, rod shaped, yellow pigmented bacterium isolated Caspian Sea, on nutrient agar plates incubated at 4 $^{\circ}C$ .The strain was positive for siderophore and HCN production. In separate invitro assays it was found to inhibit the growth and development fungi and bacteria. As agricultural production has intensified over the past few decades, producers have become more dependent on agrochemicals,for plant disease management. The increased usage of such chemical inputs has several negative effects viz., the development of pathogen resistance to the applied chemicals and the environmental impact on non target organisms (De Weger et al., 1995; Gerhardson, 2002). Furthermore, the growing costs of agro-chemicals, particularly in the lessaffluent regions of the world, and consumer demand for pesticide- free food have prompted the search for viable alternatives. In this context bacterial antagonists have been extensively explored for the control of plant pathogens.

E. acetylicum has been described as a novel alkaliphile with high catalase activity (Yumoto et al., 2004). While most reported *Exiguobacterium* strains are known for their enzyme production abilities and or extremophilic abilities (Wada et al., 2004; Kasana and Yadav., 2007), the antagonistic potential of this genus is not clearly known. The microbial diversity of the region in terms of species, richness and diversity is very high and has been little explored in the past (Pandey et al., 2006).

Antagonistic activity of *E. acetylicum* have been identified and isolated from various parts across the world (such as Caspian Sea in Iran) with antibacterial capability. *E. acetylicum* is a gram positive bacillus, arbitrary anaerobic, with low G+C % and yellow colored colonies. It can hydrolyze gelatin, stanch, casein, produced acid from fructose, maltose, raffinose and mannitol. Its major isoprene is 7- menaquinone. This microorganism is mobile with peritrichous flagellum and 1-5 mm circular colonies. Specific culture medium for these bacteria is corynebacterium agar.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The *Exiguobacterium* strains were routinely grown in tryptic soy broth (TSB, with 0.7% yeast extract at 24 or 30 $^{\circ}C$ overnight in standing cultures, as previously described.

2.2. Growth temperature range determinations

Growth at different temperatures was estimated on TSB supplemented with 1.5% agar (Difco) designated here as TSA; TSA supplemented with 0.7% yeast extract; and TSA with pH 6.8. Overnight cultures (50µl) were spotted onto plates which were then incubated at 24, 37, 42, 50 and 55 $^{\circ}C$ for 72 h.

2.2.1. Providing the strain *E. acetylicum* PTCC 1756

In present study the lyophilizied strain *E. acetylicum* PTCC 1756 was provided by microbial collection of Iranian scientific and industrial research organization as lyophilized strain and cultured in coreane bacterium broth and nutrient broth.

2.2.2. Preparing the supernatant of *E. acetylicum* PTCC 1756 bacteria culture

E. acetylicum PTCC 1756 purified in coreane bacterium broth was incubated in aerobic conditions in 37 $^{\circ}C$ to achieve a turbidity of 0.5 Mcfarland (1.5×10^{8} cfu/ml). To prepare the culture supernatant, bacterial supernatant was centrifuged for 30 minutes in 4 $^{\circ}C$ with 15000 rpm and remaining supernatant was used to examine the antimicrobial properties.

2.3. Preparing pathogenic bacteria

Four common bacterial pathogenic strain including *staphylococcus aurous* (PTCC 1431), *Escherichia coli* (PTCC 1399), *shigella dysentery* (PTCC 1188), *salmonella entrica* subspecies *Entrica* server *paratyphi* B(PTCC 1231) were provided by microbial collection of Iranian scientific and industrial research organization as lyophilized. Four strains including *staphylococcus aurous*, *Escherichia coli*, *salmonella entrica* and *shigella flexneri* were supplied by medical detection laboratory and were used in nutrient broth medium with 0.5 McFarland turbidity.

2.4. Study on the antimicrobial activity

To study on the antimicrobial activity of *E. acetylicum* PTCC 1756, Muller- Hinton agar was used. For this purpose, Disk Diffusion Agar and Well Diffusion Agar techniques were used to define MIC. Each test was performed in triplicate to decrease the error rate.

2.4.1. Well diffusion agar

During this method. Pathogenic bacteria suspension in nutrient broth (0.5 McFarland) using strile swap was cultured on Muller- Hinton agar. Then some wells were created using stirle pastor pipette. 0.1ml purified supernatant of *E. acetylicum* PTCC 1756 was poured in the wells. When medium was dried, plated were incubated in 37 $^{\circ}C$ for 24 hours. Then diameter of non growth halo created against each pathogenic bacteria was measured by a mm rule.

2.4.2. Disk diffusion agar

Paper strile disks with 6 mm diameter were soaked in supernatant of *E. acetylicum* PTCC 1756 for 5 minute. Disks were placed in 37 $^{\circ}C$ for 4 hours to be completely dried. In this technique, suspension of pathogenic bacteria cultured in nutrient broth (0.5 Mc Farland) was cultured on Muller- Hinton agar using sterile swap. Then, disks treated by supernatant of *E. acetylicum* PTCC 1756 with a definite distance from each other and from the plate edge were placed on Muller- Hinton agar. Then plated were incubated for 24 hours in 37 $^{\circ}C$. Follwingly, diameter of non growth halo created by *E. acetylicum* PTCC 1756 against each pathogenic bacteria was measured by mm ruler. Statistic analysis was performed using spss software and each test was performed in triplicate to decrease the errors.

3. Results

3.1. Biochemical examination of *E. acetylicum*

This is a positive, motile gram positive bacteria with ability to hydrolyze the starch, gelatin and casein, it is positive catalase and oxidase.

Table 1
Results of Biochemical Tests in *E. acetylicum*.

Tests	Results
Catalase	+
Oxidase	+
OF	-
Nitrate reduction	-
MR/VP	-/+
Motility	+
ONPG	+
Growth at	
5° C	-
10° C	+
20° C	+
30° C	+
37° C	+
45° C	+
50° C	+
DNase	+
Starch hydrolysis	+
Gelatin hydrolysis	+
Casein hydrolysis	+
Anaerobic growth	+

3.2. Findings achieved by well Diffusion Agar

Inhibition effect of *E. acetylicum* bacteria culture supernatant. During this technique, the highest inhibition effect was observed against the standard strain *salmonella entrica* subsp. *Entrica* server *parthyphi* PTCC 1231 with 8.5 mm inhibition and lowest inhibition rate was observed against clinical strain *Escherichia coli* with 6.60 mm.

3.3. Findings of Disk Diffusion Agar

In table 2 indicate the adequate inhibition effect of *E. acetylicum* culture supernatant. During this technique , the highest inhibition effect was observed against standard strain *salmonella entrica* sub.sp *Entrica* server *paratyphi* PTCC 1231 with 8.20 mm and the lowest inhibition rate was observed against clinical strain *Escherichia coli* with 6.50 mm.

4. Discussion

Considering the wide range of microbial diversity in several parts of the world, it is possible that concerted isolation and screening procedures could definitely throw up newer alternatives to the existing inventory of antagonistic bacteria. The genus *Exiguobacterium* includes several species that are known to possess extremophilic properties. Considering that the strain *E. acetylicum* reported in this study was isolated at 5 $^{\circ}C$ and was able to grow at temperatures ranging from 5 to 42 $^{\circ}C$, and possessed antagonistic properties, it would be appropriate to call it‘‘cold tolerant antagonistic bacterium. We observed that itwas for positive DNAse and negative lecithinase activities, which are potential biosafety indicators for a bioinoculant. It was indeed surprising to observe that the *bacterium* was able to inhibit the growth and development of four major plant pathogenic species in three separate assays. While the highest inhibition was observed in the volatile compound assay across the pathogenic fungal species tested, this bacterium was isolated from Caspian sea coastal waters in 2009 and was identified by

using PCR. This bacterium has a high antagonistic power compared to *Salmonella typhi*، *Shigella flexneri*, *Staphylococcus aureus*, *Escherichia* coli (Javanbakht et al., 2009). In the study of (Yumoto et al., 2004), *E. oxidotolerans* sp. was isolated from the plants surrounding the water. This bacterium is alkalophile and displayed high catalystic power. Its growth ability ranged 7 to 10 (Yumoto Hishinuma-Narisawa, 2004). In the study by (Selvakumar et al., 2009) , *E. acetylicum* 1P was defined from the soil of Himalian mountains in north western India. In this study also supernatant of *E. acetylicum* culture displayed a good antimicrobial property.The greatest effect antimicrobial property. The greatest effect was observed during well diffusion technique and on the standard strain of *salmonella enteric* sub.sp *Enterica* server *paratyphi* PTCC1231 with growth inhibition diameter of 8.5 mm.

5. Conclusion

In conclusion, supernatant of these bacteria has greater inhibition ability on the standard strains compared to clinical strains. Also with increased antibiotics resistance and outcomes of chemical drugs consumption. It is necessary to use alternative drugs. *E. acetylicum* supernatant having a great antimicrobial ability and having useful compounds with inhibitory property, may be considered as an antimicrobial factor.

Table2
Antimicrobial activity of bacterial supernatants *E. acetylicum* against standard strains and clinical well Diffusion Agar in mm.

supernatants / **Standard and Clinical Strains**		**Mean of**	**Mean of (3 times)**
Escherichia coli PTCC 1533	7 6.5 7.5		7
Salmonella enterica PTCC 1231	9 8.5 8		8.5
Staphylococcus aureus PTCC 1112	7 7.5 7.2		7.23
Shigella dysenteriae PTCC 1234	9 8 8		8.33
Escherichia coli	7 6.5 6.3		6.60
Salmonella enterica	8.5 8 7.5		8
Staphylococcus aureus	7 6.5 7.5		7
Shigella dysenteriae	8 7.5 7.5		7.66

Table 3
Antimicrobial activity of bacterial supernatants *E. acetylicum* against standard strains and clinical Disk Diffusion Agar in mm.

Supernatants / Standard and Clinical Strains	Mean of	Mean of (3 times)
Escherichia coli PTCC 1533	7 6.5 7.5	7
Salmonella enterica PTCC 1231	7.8 8.5 8.3	8.20
Staphylococcus aureus PTCC 1112	7 6.5 7	7.16
Shigella dysenteriae PTCC 1234	8 7.5 7	7.50
Escherichia coli	6 7 6.5	6.5
Salmonella enterica	8 7.5 7.2	7.56
Staphylococcus aureus	6.5 7 7	6.83
Shigella dysenteriae	7 6.5 7.5	7

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