In vitro evaluation of bioactive potential of *Bacillus methylotrophicus* YML008 against *Propionibacterium acnes*

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Abstract: *Acne vulgaris* is the most common skin diseases that people experience during their lives. Thirteen rhizosphere isolates were screened against *Propionibacterium acnes*. The bacterium exhibited the highest activity against *P. acnes* was identified as *Bacillus methylotrophicus* YML008 by 16S rRNA gene sequencing. Scanning electron microscopy was used to assess the changes in morphology of *P. acnes*. Preliminary studies on the antimicrobial substance demonstrated the hydrophilic nature of compound with MIC of 0.17mg/ml and MBC of 0.3mg/ml. The cytotoxic effect of the extract was least (80% survival) as compared to benzyperoxide (40% survival). These results suggest YML008 as a promising bioresource and may be useful as a lead bacterium to develop a new type of anti-acne skin care prep to cure or prevent acne. Further, mechanism of action and proper clinical trials may be promising for this research.

Keywords: Acne, inflammation, skin disorder, antimicrobial, antibiotics

INTRODUCTION

The skin is always in contact with a variety of environmental factors responsible for skin disease. Direct exposure to oxidative stress, ultraviolet (UVA) radiations (David and Mohammad, 2006; Girard *et al*., 2011), visible light (Papageorgiou *et al*., 2000), combination of UVA and visible light (Meffert *et al*., 1990), chemical and physical pollutants (Athar, 2002), and microbial invasion result in respiratory and inflammatory responses (Farrar *et al*., 2000). Microorganisms are the main cause of such inflammatory reactions leading to skin irritation. Therefore, skin infections caused by microorganisms are known for decades to be more annoying, and acne is of major concern also caused by strains of Propionibacteria (Loveckova *et al*., 2002). Although, acne is not of serious concern, the quality of life in patients might get affected during adolescence and adulthood (Ozolins 2005; Krautheim 2004).

At present, accessible topical treatments for acne lesions are the sedate that show efficiency until treatment is continued. Though, vitamin A-derived retinoid is mostly used for the treatment of severe cystic acne vulgaris (James *et al*., 2000); however, the medication is associated with serious side effects (Oda *et al*., 1996).

For many years, the strains of genus Bacillus are used in food and fermentation industries. Currently, more than half of today's commercial production depends on the application of Bacillus strains that produce diverse range of enzymes, antibiotic, and/or antimicrobial substances (Schallmey *et al*., 2004). In this study, anti-acne mode of action of *B. methylotrophicus* was evaluated against *Propionibacterium acnes* KCTC3020.

MATERIALS AND METHODS

Isolation, Identification and Characterization

Bacillus methylotrophicus YML008 was isolated from soil using MRS agar or in MRS broth (Difco laboratories, USA) at 37ºC for 24 h. The 16S rRNA gene sequencing was carried for strain identification. A method described by Lane (1991) was used to amplify the 16S rRNA gene by PCR using universal primers (Rather *et al*., 2013).

Preliminary antimicrobial activity analysis

Antimicrobial mode of action of isolate *B. methylotrophicus* YML008 against *P. acnes* KCTC3020 was carried out using paper disc assay. A 30µL of sample was used to impregnate on 8 mm paper discs (Advantec Roshi Kaisha, Ltd, Tokyo, Japan). The disc was then placed on the agar plate already spread with indicator the strain. After incubation, the zone of inhibition (mm) around paper disk was measured. The significantly higher activity above 20 mm diameter was analyzed statistically by one-way analysis of variance (ANOVA).

Characterization of antimicrobial compound

The cell-free-supernatant of YML008 strain was treated with different enzymes (1mg/mL) in order to check the nature of the antimicrobial compound. Briefly, the supernatant was treated with trypsin, proteinase K, alpha-amylase, lipase, and catalase, and the mixture was incubated at 37°C for 3h. To inactivate the enzyme

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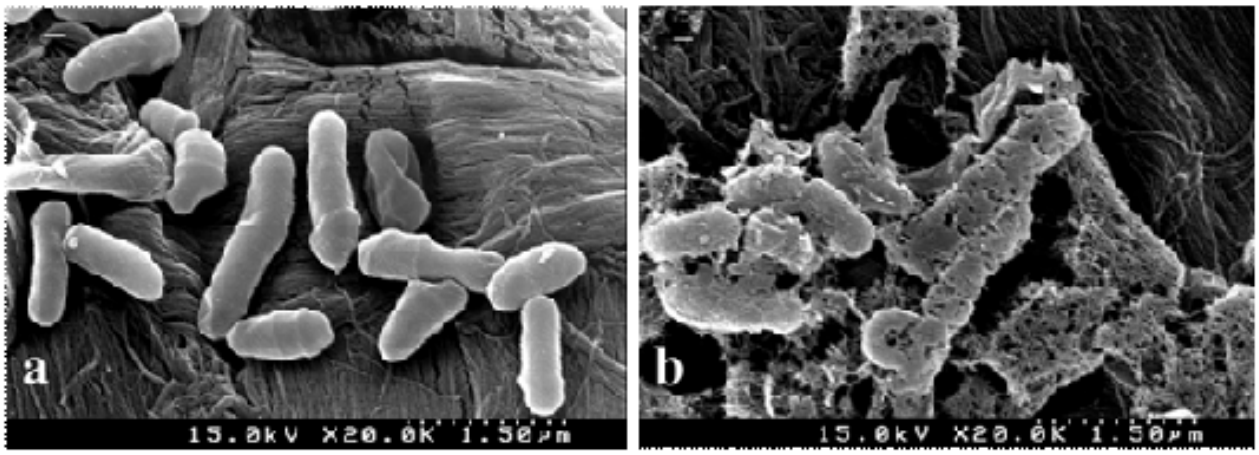

Fig. 1: Scanning electron microscopy photographs of *Propionibacterium acnes:* (a) Control without any treatment and (b) Test, cells treated with crude extract of *B. methylotrophicus* YML008.

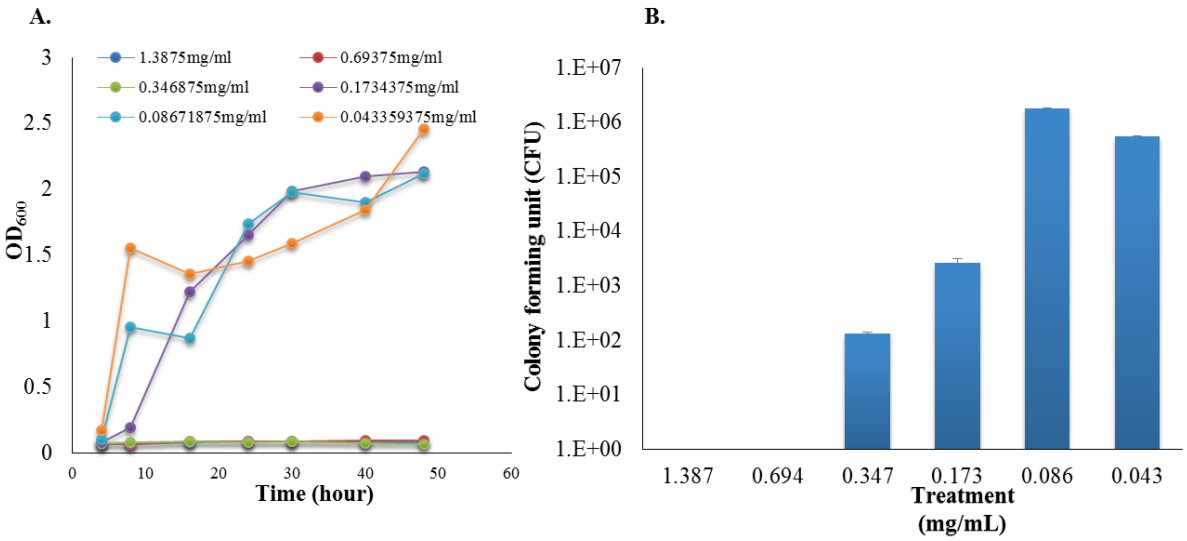

Fig. 2: Minimum inhibitory concentration (A), and Minimum bactericidal concentration (B) of the extract from YML008.

activity, the mixtures were cooled and subsequently analyzed for antimicrobial activity against *P. acnes* using paper disc method. Further, to examine the nature of the antimicrobial compound, 20 liter of sterilized cell-free-supernatant of *P. acnes* was treated with equal volume of 90% ethanol and incubated for 48 hours. The extract was dried under reduced pressure in a rotary evaporator at 45°C and stored at 4°C for further experiments. The experiment was carried under sterile conditions to avoid any contamination.

Scanning electron microscopy analysis

Scanning electron microscopy (SEM) was done to determine the morphological characteristics during inhibition of *P. acnes*. Using a polylysine-coated glass slide, the *P. acnes* suspension and extract of *B. methylotrophicus* YML008 strain was spotted. A solution of 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) was used to fix the slide followed by fixation in 1% osmium acid solution for 2h (Rather *et al*., 2013). The sample dehydration was done using 30% ethanol for 10 min with 1~2 repeats, further dehydration was done using 50%, 70, 80, 90, 95 and 100% ethanol each for 10 min (Rather *et al*., 2013). With critical point drying (Hitachi, HCP-2, Japan), the sample was dried using CO_2 as transition fluid. At final stage, the slide was tapped onto stubs, gold sputter coated and observed using SEM (Hitachi, JEOL, Japan). For comparison, control slide containing only *P. acnes* was processed in similar fashion. The reagents used were purchased from Sigma-Aldrich, USA.

Cytotoxicity test

To check the cytotoxicity effects of the extract isolated from *B. methylotrophicus* YML008 strain, Dulbecco's Modified Eagle's Medium (DMEM) was used to grow human dermal fibroblast cells supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine, 100 unit/ml

penicillin and 100ug/ml streptomycin. A fully humidified atmosphere was maintained under 5% CO_2 and the cells were incubated at 37°C (Sabudin *et al*., 2012). Further, the ethanol extract and rhodo-myrtone were dissolved in DMSO at a final concentration of 100 and 20mg/mL, respectively. The samples were serially two-fold diluted in the culture medium. The two-fold diluted samples were then added in cell culture and incubated at 37°C for 24 h in the presence of 5% CO_2 using CO_2 incubator (Sabudin *et al*., 2012). After 24h incubation, the medium was replaced with fresh medium and re-incubated for another 24h. MTT assay was carried out to check the cell viability. Using 96 well-plate, MTT (5mg/mL in PBS) was instilled into each well followed by 4 hours of incubation and OD_{570} was measured.

RESULTS

Isolation and characterization of Bacillus methylotrophicus YML008

In the present study, a total of thirteen bacterial isolates were isolated from different rhizospheres. The samples were collected from different locations in South Korea following the standard protocols. Finally, based on the preliminary antimicrobial screening against *P. acnes*, a potent skin pathogen, *B. methylotrophicus* was selected for further evaluation. The rhizosphere bacterial strain was further identified and characterized as *B. methylotrophicus* YML008 based on the 16S rRNA gene sequencing with GenBank accession number of JQ277696.

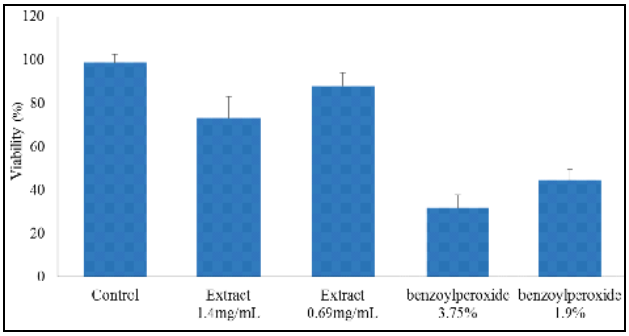

Fig. 3: Effect of crude extract from *B. methylotrophicus* YML008 and benzoyl peroxide on cell viability.

Antimicrobial efficacy of cell free supernatant of Bacillus methylotrophicus YML008

The antimicrobial efficacy of *B. methylotrophicus* YML008 cell-free supernatant was tested against *P. acnes*. Interestingly, *B. methylotrophicus* YML008 exhibited a significant ($p<0.05$) inhibitory effect against *P. acnes* with highest zone of inhibition of 21.7±0.2mm. However, it was interesting to observe that the antimicrobial activity of *B. methylotrophicus* YML008 was not affected by enzyme treatment.

Scanning electron microscopic analysis

The antimicrobial assays showed *B. methylotrophicus* YML008 strain to harbor portent antibacterial activity again *P. acnes*. Specific antimicrobials may cause changes in cell wall of specific bacteria. Therefore, SEM was performed to visualize the effect of the crude extract of *B. methylotrophicus* YML008 on the morphology of *P. acnes* cells. The cells of *P. acnes* in the absence of treatment showed a steady and smooth surface (fig. 1a). In contrast, *P. acnes* cells treated with the crude extract of *B. methylotrophicus* YML008 (MIC 0.17mg/mL) revealed severe damaging effect on the cell morphology, disruption of cell membrane as well as abnormal cell breakage, and cell lysis (fig. 1b).

Minimum inhibitory concentration of the crude extract from B. methylotrophicus YML008

To confirm the bioactivity potential of the extract of *B. methylotrophicus* YML008, the extract was evaluated for its minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values in order to further confirm its efficacy and cytotoxicity. Accordingly, the extract isolated from *B. methylotrophicus* YML008 showed appreciable antibacterial activity against *P. acnes* with MIC and MBC values of 0.17mg/mL and 0.3mg/ml, respectively (fig. 2). Earlier, some other researchers also reported the susceptibility of other types of bacterial pathogens in terms of their MIC values when treated with different antimicrobials of plant and microbial origin (Bajpai *et al*., 2010; Lee *et al*., 2013).

Cytotoxicity assay

The ethanol extract procured from the whole cell culture of *B. methylotrophicus* YML008 was evaluated for its cytotoxicity against human fibroblast cells compared to the standard cytotoxic agent benzoyl peroxide. The results from cytotoxicity assay confirmed that the extract containing antimicrobial compound from *B. methylotrophicus* YML008 has remarkable effect on the cell viability of human fibroblast cells at the tested concentrations as compared to the control. The extract had significantly reduced number of viable human fibroblast cells of post treatment when compared with the control (fig. 3 and 4). Cytotoxic effects of some purified compounds such as limonoids have been reported previously (Poulose *et al*., 2005; Souto-Lopes *et al*., 2013).

DISCUSSION

A number of studies on antimicrobial activity of various bacterial strains against skin infection causing bacteria have been reported earlier (Kang *et al*., 2009; Kang *et al*., 2012; Itoh *et al*., 2014). Although, in these studies, the bacteria belonging to different genera showed appreciable inhibitory effect against the growth of *P. acnes*; however, the inhibitory effect was less pronounced compared to *B.*

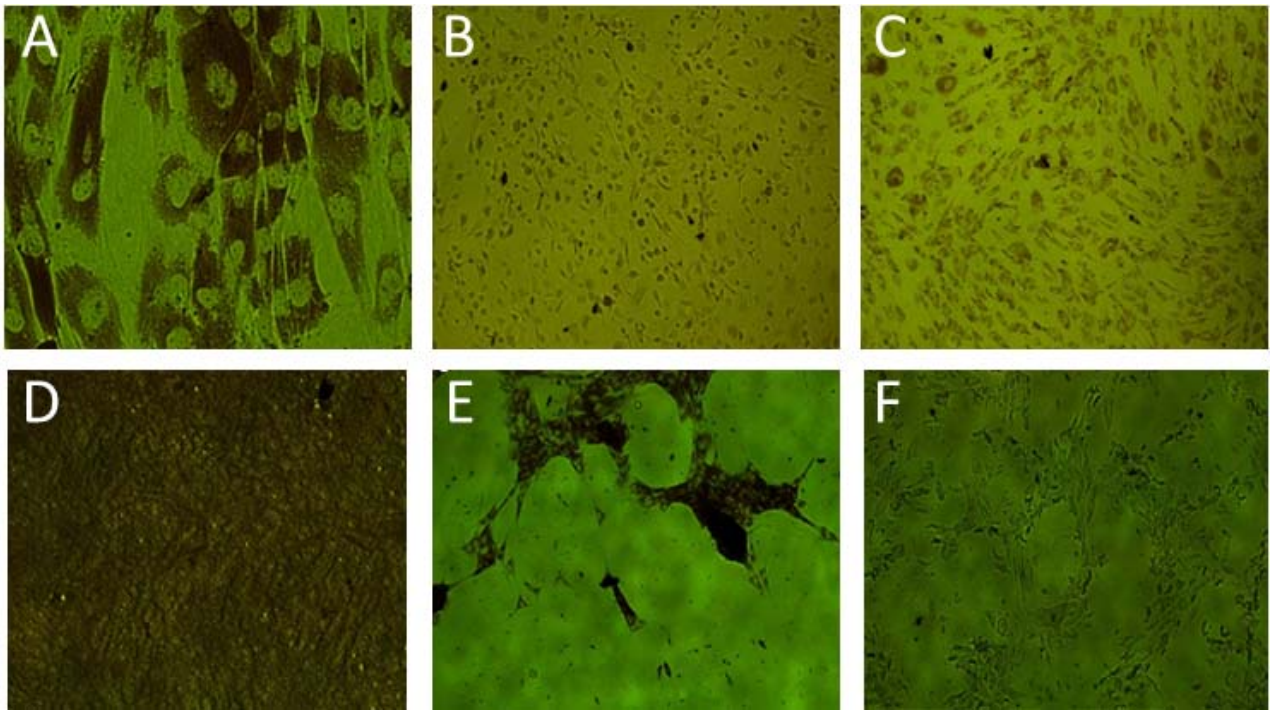

Fig. 4: Cell cytotoxicity test using human fibroblast cells. (A) Control; (B) Treated with 1.4mg/mL crude extract of YML008 strain; (C) Treated with 0.69mg/mL crude extract of YML008 strain; (D, E, F) Treated with 7.5%, 3.75% and 1.9% of Benzoyl peroxide, respectively.

methylotrophicus YML008. The antimicrobial activity of YML008 strain might be related to different biological compounds which might be antagonistic in terms of their ability to inhibit the growth of the test pathogens as also reported earlier (Mezaini and Bouras 2013). Electron microscopic analysis revealed that the crude extract of *B. methylotrophicus* YML008 had significant inhibitory effect on the morphology of *P. acnes*. However, it is hard to predict the mechanism of inhibition, the changes in morphology could be because of the effect of crude extract of *B. methylotrophicus* YML008 on the membrane integrity. Similar findings were reported previously against different pathogenic bacteria and fungi (Rather *et al*., 2013; Bajpai *et al*., 2015). In addition, the cytotoxicity of crude extract of *B. methylotrophicus* YML008 was less as compared to the Benzoyl peroxide. These results reinforce *B. methylotrophicus* YML008 as a novel bacterium for the prevention and treatment of skin infection.

CONCLUSION

This study evaluated the potential effect of *B. methylotrophicus* YML008 that inhibited the growth of one of the important skin pathogens *P. acnes* KCTC3020 *in vitro*. The results reinforce the suggestion that the extract derived from *B. methylotrophicus* YML008 might have significant potential to serve as potent bioactive agent for the treatment of skin infections.

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