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Original research article

Screening of Biosurfactant-Producing Bacteria as a Potential Biological Control Agent for Fungal Orchid Pathogens in Thailand

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ABSTRACT

In southern Thailand, biosurfactant-producing bacteria were isolated from palm oil mill facility soil using serial dilution. Palm oil mill effluent (POME) was utilized as the sole carbon source. A total of 231 oil-degrading bacterial isolates were discovered from 40 samples. Based on the examination of surface tension reduction and emulsification activities, 30 isolates showed promising biosurfactant activities; however, only 85 of the isolates tested positive for the formation of biosurfactants based on the oil displacement test. The biosurfactants from 30 bacterial isolates were tested for their antifungal efficacy against *Phytophthora palmivora* Al2 using an agar well diffusion assay. The strongest antifungal activity was seen in the biosurfactants produced from strain CT03. Analysis of the bacteria's 16S rRNA gene revealed the strain to be *Acinetobacter calcoaceticus*. It was discovered that MSM containing $(NH_4)_2SO_4$ and glucose acting as carbon and nitrogen sources, respectively, provided the best conditions for *A. calcoaceticus* CT 03 to produce biosurfactants. A combination of chloroform: methanol (2:1, vol/vol) was used to extract 1.75 g/L of biosurfactant. The structures of the resulting biosurfactant compounds was determined using three distinct methods of spectroscopy: Fourier transform infrared (FT-IR), nuclear magnetic

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resonance (NMR), and mass spectroscopy. Additionally, the biosurfactants created by *A. calcoaceticus* CT 03 contained molecules of a lipopeptide that resembled surfactin. This study indicates the ability of this biosurfactant mixture to prevent pathogenic fungal growth in orchids.

Keyword: *Acinetobacter calcoaceticus*; Biosurfactant; Orchid disease; *Phytophthora palmivora*

1. Introduction

Out of a variety of tropical beauty crops, the orchid, in the form of cut flowers, is the most expensive to produce in Thailand [1]. Orchids make up one of the world's largest families of blooming plants, with over 700 different genera and almost 28,000 species [2]. Orchids are a prized plant among collectors and cater to a wide range of interests due to their wide variety of forms, sizes, and hues. Because of this, they are undoubtedly recognized as an economically significant ornamental crop in the global floriculture business, both as cut flowers and as potted plants [3,4]. Dendrobium orchids have gained more and more renown among the numerous orchid genera [4]. Despite being Thailand's main export in cut-flower orchids, they frequently struggle with disease. Due to low productivity brought on by plant diseases, the orchid sector is experiencing financial difficulties. Fungi are the main contributors to orchid infections, which cause a severe drop in productivity and financial losses for farmers [5, 6]. Because of this, it is crucial to maintain disease control [7]. The water mold *Phytophthora palmivora*, which infects many different kinds of orchids, is one species that causes black rot disease. The base or roots of the pseudobulbs of infected orchids initially displays small black lesions, which subsequently grow to completely encircle the pseudobulbs and leaves [8, 13].

Numerous diseases in orchids are brought on by fungal and mold pathogens, including rust spot disease on the flowers caused by *Curvularia lunata* [9], rusty flower disease caused by *C. eragrostidis* [10], column blight disease caused by *Fusarium moniliforme*, leaf yellowing caused by *Fusarium solani*, root and collar rot diseases caused by *Fusarium solani* [11], wilt disease caused by *Fusarium oxysporum*, collar rot disease caused by *Sclerotium rolfsii*, flower spot disease caused by *Phoma exigua*, anthracnose disease caused by *Colletotrichum gloeosporioides* [12], and black rot disease caused by *Colletotrichum gloeosporioides* [9]. *P. palmivora*, which is recognized as a water mold, causes the black rot disease that affects a wide variety of orchids, including Aerides, Ascocenda, Brassavola, Dendrobium, Gongora, Miltonia, Maxillaria, Oncidium, Paphiopedilum, Phalaenopsis, Rhynchostylis, and Schomburgkia [13]. The orchid succumbs to the mold once it has spread to other areas of the plant. Numerous Phytophthora species have been linked to widespread reports of economic harm to orchids. A rare, polyphagous pathogen (*Phytophthora palmivora*) that causes black rot has been observed on a variety of hosts throughout the world and is mostly responsible for the diseases known as root and crown rot. It has spread widely in Asia and Europe, where reports of it occurring in a variety of ornamental plants, including orchids, have been made [14, 15]. The prevalence of these diseases has grown in recent years due to polyphagy and dissemination methods. Additionally, because of its thermophilia, it thrives in hot climates like that found in Thailand [16]. Infected plants can be killed very quickly because the disease can spread throughout the rhizomes [17, 18].

Many studies have discussed the use of chemical control methods to inhibit *Phytophthora* infections. When chemicals are used in the field, soil insects and beneficial rhizosphere microbes commonly die off. Due to the negative effects on the environment,

human health, and the emergence of fungicide-resistance. synthetic fungicides should not be used on crops. As chemical control methods have their drawbacks, biologically friendly control methods utilizing naturally occurring microbes have been intensely researched [19-22]. After recently demonstrating their effectiveness as strong antifungal agents, biosurfactants were investigated for possible crop protection applications. The antifungal activities of lipopeptide biosurfactants are widely established [23-25] Iturin, surfactin, lichenysin, and mycosubtilin were discovered to have surface-active properties and antifungal activity [26-29]. Surfactin and iturin both include an amino fatty acid [30, 31]. Previous research has shown that lipopeptides as mycobacillin, iturin, and surfactin have antifungal activities that are crucial for plant defense [32, 33]. The use of antifungal lipopeptide biosurfactants has been found to have biocontrol effects against a number of fungal plant pathogens [34, 35]. Having the ability to penetrate the target pathogen's cell membrane is necessary for a biosurfactant to be fungitoxic [31, 32, 36]. A number of biosurfactant pathways are responsible for the disruption of the pathogen's cell surface and membrane [37]. It was found that *Pseudomonas*-generated biosurfactants isolated from agricultural plants had greater antifungal effects against *Phytophthora capsici* infecting pepper plants, when compared to two fungicide treatments, including acibenzolar-S-methyl (ASM) and ASM + mefenoxam [38]. On the other hand, little research has been done on the effect of biosurfactant-producing strains on *Phytophthora* species found in orchids.

The aim of this study was to characterize biosurfactants produced by bacteria isolated from oil-contaminated soil gathered from four different palm oil mill plants and to evaluate their potential for use in inhibiting orchid *Phytophthora* infection.

2. Materials and Methods

2.1 Soil samples, biosurfactant-producing bacteria isolation and identification.

In Southern Thailand, four different palm oil mill plants each contributed ten samples of oil-contaminated soil, from which the biosurfactant-producing bacteria were identified. On MSM agar plates, the samples were serially diluted and distributed with the following (g/L): 0.8 K_2HPO_4, 0.2 KH_2PO_4, 0.05 $CaCl_2$, 0.5 $MgCl_2$, 0.01 $FeCl_2$, 1.0 $(NH_4)_2SO_4$, and 30 NaCl [39]. Bacteria were isolated using MSM agar with 1% (w/v) POME as the carbon source. Gram-staining was then carried out after purified cultures were obtained by re-isolating morphologically dissimilar colonies by moving them onto new POME-containing agar plates at least three times. Nutrient broth (NB, Difco, MI, USA) was combined with sterile glycerol at a final concentration of 30% (v/v) and refrigerated at -20 °C for pure cultures.

2.2 Determination of potential biosurfactant-producing strains

The biosurfactant-producing strains were discovered and reported by Saimmai et al. [40]. In brief, selected strains were cultivated in NB and incubated for 24 hours at 200 rpm and 30 °C. A 5% portion of the cell culture was transferred to MSM media that had been supplemented with 1% (w/v) POME and was then grown in a rotary shaker (Vision Scientific Co., Daejon, Korea) for 48 hours at 30 °C. Next, 1% POME-supplemented MSM was used as a negative control. The drop-collapsing test, oil displacement test, emulsification activity, and surface tension of the culture supernatant were examined [41] after it had been extracted three times with the same volume of hexane and centrifuged for 10 minutes at 8,500 rpm at 4 °C. The oil displacement test, developed by Morikawa et al. [42], involved placing 30 ml of distilled water in a Petri dish and adding 1 ml of lubricating oil to the center. Then, 20 µL of the bacterial culture supernatant was added to the oil layer. Distilled water served as the negative

control, while a 10% (w/v) SDS solution served as the positive control. The test involved observing the Petri dishes closely for the appearance of a displacement zone in the oil, and the diameter of the displacement zone was subsequently measured. The 20 bacterial strains that exhibited the highest biosurfactant activity were chosen for additional study.

2.3 Isolation, identification and preparation of spore suspension of Phytophthora

P. palmivora (Butl) was isolated by tenfold dilution from 30 infected tissue samples of orchids collected in Phuket, Krabi, Phangnga, and Trang provinces and then spread on potato dextrose agar (PDA; HiMedia, Mumbai, India). In preparation for further research, the fungus was harvested and cultivated on PDA slants for five days at 37°C. The entire DNA of *P. palmivora* (Butl) was extracted using the traditional phenol-chloroform technique [43].

According to Dlauchy et al. [44], 18S rDNA was amplified with the nearby ITS1 region using the NS1 (5′ GTAGTCATATGCT TGTCTC 3′) and ITS2 (5′GCTGCGTTCTTC ATCGATGC3′) primers. The nucleotide sequence was verified using BLAST analysis. Over the course of 5-7 days, *P. palmivora* (Butl) was cultured on PDA slants at 37 °C. After the incubation period, the spores were suspended in sterile 0.1% (v/v) Tween 80 and diluted with Potato Dextrose Broth (PDB) to a concentration of 106 spores/mL, as assessed by hemacytometer (BOECO, Germany) [45].

2.4 Antifungal activity of biosurfactant

An agar diffusion experiment was used to test the extracted biosurfactant's antifungal activity after it had been weighed and diluted in distilled water at a concentration of 10 mg/mL. A 5 mm diameter PDA well that had already been seeded with the *P. palmivora* spore solution (10^6 spores/mL) was punched to receive the sterile extracted biosurfactant (10 µL). Negative control wells were filled with 10 µL of distilled water and the positive control wells were filled with 10 µL of 64% mancozeb. The antifungal and control wells were spread in triplicate across the infected agar plate's surface. After 48 hours of incubation at 37 °C, the well's inhibitory zones were measured with a vernier caliper, and the average diameter of the zones was recorded. The strains with the greatest antifungal activity were chosen for further research.

2.5 Identification and phylogenetic relationship of biosurfactant-producing bacteria

A DNA Purification Kit (NucleoSpin gel and PCR clean-up, Germany) was used to purify the DNA extracts after the genomic DNA from 5 g of samples was isolated using the MagNA Pure LC DNA isolation kit in accordance with the manufacturer's instruct-tions. DNA concentration was measured using a Nano Drop 1000 UV-Vis spectrophotometer (USA) calibrated to 260/280 nm. Isolated genomic DNA was stored at -20 °C pending PCR analysis. PCR was used to amplify the 16S rDNA gene (PerkinElmer 2400; USA). The bacterial 16S rDNA gene was amplified using the primers UFUL-f (5′-GCCTAACACATGCAAGTCG A-3′) and URUL (5'-CGTATTACCGCGGC TGCTGG-3') to produce 1,500 bps of PCR product in highly conserved regions. 1.5% (w/v) agarose gel electrophoresis at 100 V for 25 minutes was used to validate the PCR results. Bands were spotted using ethidium bromide staining, and the gel was subsequently stored at -20 °C for nucleotide sequencing. The NCBI's (National Center for Biotechnology Information) BLAST tool was used to compare the nucleotide sequences to those in the GenBank database. A phylogenetic tree was produced using Molecular Evolutionary Genetics Analysis (MEGA) Software Version 6.0 and the neighbor-joining algorithm [46].

2.6 Factors influencing antifungal activity of *A. calcoaceticus* CT 03

In a series of tests, variables affecting *A. calcoaceticus* CT 03's antifungal activity were changed one at a time while the other

variables remained fixed under a particular set of circumstances. In order to increase the antifungal activity of the produced biosurfactant, eight variables were studied: carbon source, carbon concentration, nitrogen source, nitrogen concentration, inoculum concentration, agitation speed, initial pH, and incubation period. The carbon sources employed were palm oil, used palm oil, soybean oil, POME, molasses, glucose, and sucrose. The carbon content ranged from 0.5% (w/v) to 5% (w/v). The nitrogen sources employed were Peptone or yeast extract, beef extract, commercial monosodium glutamate (CMSG), $NaNO_3$, $(NH_4)_2SO_4$, NH_4Cl, and NH_4NO_3. Between 0.5% and 3% (w/v) of the sample was nitrogen. A 50-300 rpm range was used for the agitation speed. The pH of the medium was changed to be between 5.0 and 8.0 in order to assess how pH and temperature affected the generation of biosurfactants. The surface tension of the biosurfactant that the bacterial strain produced was measured using a Fisher Science Instrument Co. Model 20 Tensiometer, Pittsburgh, Pennsylvania, USA [18].

2.7 Recovery of biosurfactant

After centrifugation of the culture broth at 12,000 g for 15 min, culture supernatant was obtained. Four solvent systems, which included a mixture of chloroform: methanol (2:1) [39], cold acetone [7], dichloromethane [15], and ethyl acetate [33], were tested for biosurfactant extraction in order to determine the most efficient method. The culture supernatant was extracted three times using an equal volume of the solvent system, followed by evaporation under vacuum. The crude biosurfactants were then dissolved in distilled water, and the biosurfactant activity was measured. The method that exhibited the highest antifungal activity was chosen for the recovery of biosurfactant from *A. calcoaceticus* CT 03.

2.8 Biosurfactant characteristics

Fourier transform infrared (FT-IR) spectroscopy was used in conjunction with the KBr pellet method to characterize the biosurfactant [47]. The dried biosurfactant samples (0.3-0.5 mg) were ground in about 80 mg of spectral grade KBr (Merck, Darmstadt, Germany), and then pellets were formed using a hydraulic press (Specac, Orpington, Kent, UK) at a pressure around 6 t/cm^{-2}. A GX-FT-IR system (Perkin-Elmer, Norwalk, Connecticut) was used to record the infrared absorption spectra. On an AMX 300 NMR spectrometer (Bruker, 300 MHz) at 298 K, a spectrum of 1H and 13C nuclear magnetic resonance (NMR) was captured. The Aspect 3000 computer (Bruker) was installed, and it was locked, without spinning, to the CDCl3 solvent's deuterium resonance. A 32 K (the number of data points per parts per million of the plot) resolution was used for data recording [48]. The biosurfactant was analyzed using electrospray ionization mass spectrometry (ESI-MS) in a Finnigan MAT LCQTM quadrupole ion-trap mass spectrometer, which makes use of electrospray ionization (ESI). The electrospray source was run at 80 °C for the ionization source, 200 °V for the electrolyte, and 120 °C for the spray inlet. The apparatus was operated in positive ion mode [40].

3. Results and Discussion

3.1 Isolation and screening of biosurfactant producing bacteria

Samples collected from various locations inside the palm oil mill factory in southern Thailand produced a total of 231 strains using the MSM medium supplemented with 1% (w/v) POME as the carbon source. Differences in sampling sites have an impact on the resident bacteria's capacity to create surfactants because of the soil's composition, nutrients, and length of oil deposition [49-51]. Both the ability to make biosurfactants and the use of POME as a carbon source by these strains have been demonstrated. During the initial screening, only 85 of the positive

bacterial strains demonstrated the formation of an oil-displacement halo around the colonies. The visible effects of the biosurfactants produced by different isolated bacteria are shown in Fig.1.

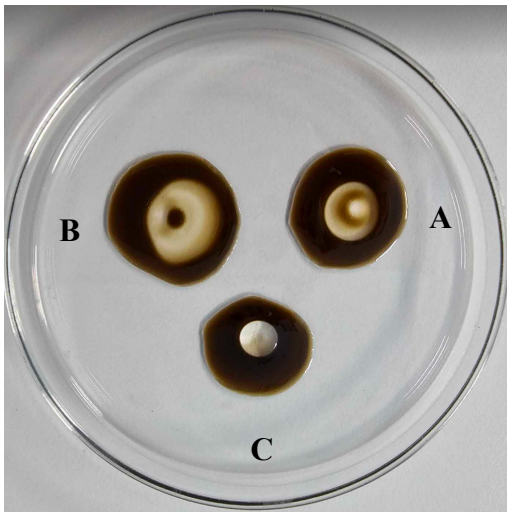

Fig. 1. Oil displacement test comparing the biosurfactant generated by biosurfactant-producing microorganisms (A), along with positive (B) and negative (C) control.

On MSM medium supplemented with POME and glucose as the only carbon source, 30 bacteria displayed high optical density (OD600), ranging from 0.19 to 3.87, and these strains were chosen for further investigation. The isolates of LT02 and CT01 from Lapthavee Palm Oil Co., Ltd. and Saha Rungsab Palm Oil Co., Ltd., respectively, showed the highest OD600 values (2.98-3.87). The 21 isolates of this bacterium were Gram-negative and had short rod morphologies. The majority of the bacteria in soil-contaminated oil were Gram-negative, according to earlier investigations [52]. Gram-negative bacteria have been shown to be more resistant to stress than Gram-positive bacteria [53, 54]. The culture supernatant's surface tension, antifungal activity, dry cell weight, and emulsifying activity (EA) were all measured 48 hours after the initial screening. When grown on glucose and POME, the strains PO02 and CT03 from Purmpoonsub Palm Oil Limited Partnership and Saha Rungsab Palm Oil Co., Ltd. had the greatest decrease in surface tension in the 22.89 - 23.50 mN/m range. The strain PO01 displayed the highest EA (50.16-60.88%).

The isolated biosurfactants can be generally categorized into two different groups according to their molecular weight [55, 56]. Low molecular weight biosurfactants include fatty acids, glycolipids, lipopeptides, lipoproteins, neutral lipids, and phospholipids. These molecules efficiently reduce surface and interfacial tension [57, 58]. High molecular weight polymeric biosurfactants include polysaccharides, glycoproteins, lipopoly-saccharides or mixtures of these macromolecules [59, 60]. Although their primary purpose is to stabilize two immiscible liquids, such as hydrocarbons or other hydrophobic substrates, these surfactants are ineffective at lowering surface tension [61, 62].

3.2 Isolation and identification of *P. palmivora*

Seven isolates were shown to have 99-100 % similarity to *P. palmivora* after culturing of the diseased orchid tissues on PDA medium. As shown in Fig. 2, all strains of *P. palmivora* have close connections to numerous fungal diseases in orchids according to phylogenetic research. *P. palmivora* develops as a whitish, loosely matted colony on PDA medium (Fig. 3). Growing *P. palmivora* on PDA yields results that are comparable to those previously reported [1]. Previous investigations have shown that *P. palmivora* is more aggressive and destroys even bigger roots than other Phytophthora species [63].

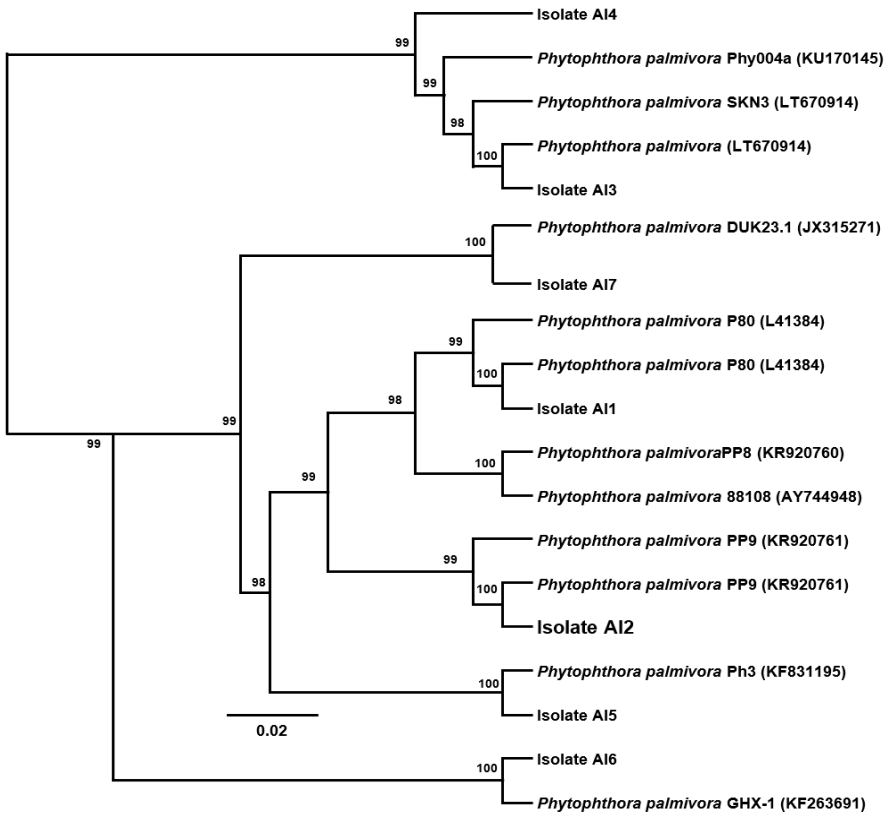

Fig. 2. Phylogenetic tree based on neighbor-joining of full *P. palmivora* 16S rDNA sequences showing the relative locations of biosurfactant isolates AI1, AI2, AI3, AI4, AI5, AI6 and AI7. The nodes of the tree display the bootstrap values resulting from a total of 100 repetitions.

Fig. 3. After 2 days of incubation, *Phytophthora palmivora* (Butl.) morphological features on PDA.

3.3 Antifungal activity of biosurfactant-producing bacteria

An agar diffusion experiment on PDA medium was used to examine the antifungal effectiveness of 30 biosurfactant-producing bacterial strains. Most of the strains displayed inhibition at 5 to 10 mm. The largest inhibition areas (10.46 cm) against isolate CT 03 were shown by *P. palmivora* AI1, AI2, AI3, and AI4 (Fig. 4). Inhibition was absent in the strains CT 04, KB 03, NH 02, PM 01, PO 02, and RJ 01. In this regard, numerous investigations have discovered that a variety of fungi can be suppressed by biosurfactant-producing bacterial strains [64, 65]. Joshi et al. found that *Alternaria burnsii*, *Chrysosporium indicum*, *Fusarium oxysporium*, *Fusarium udum*, *Rhizoctonia bataticola*, and *Trichoderma herzanium* were

all inhibited by a *Bacillus subtilis* 20B strain that produced biosurfactants [66]. Different fungus strains were suppressed by a number of biosurfactants made from bacteria in oil polluted by dirt [67-70].

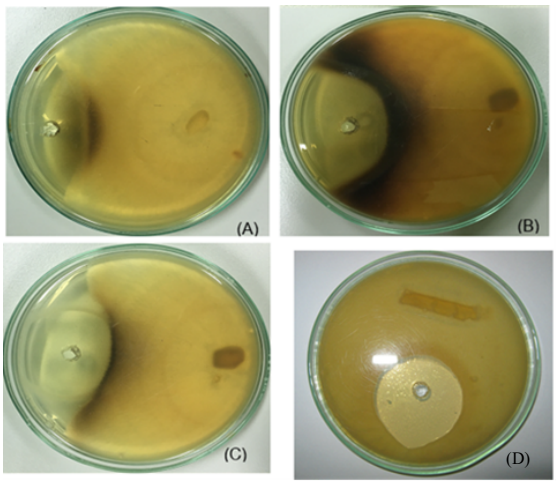

Fig. 4. After 7 days of incubation, antifungal activity of biosurfactant generating strain CT 03 against *P. palmivora* AI1 (A), AI2 (B), AI3 (C), and AI4 (D).

3.4 Identification of biosurfactant producing bacteria

Due to its ability to control fungal infections and its ability to lower surface tension more than other strains, strain CT 03 of the isolated bacteria was used in earlier studies. *Acinetobacter calcoaceticus* PA (GenBank accession number KT878384) and CT 03 have 100% identical 16S rDNA nucleotide sequences. Previous studies have identified *Acinetobacter* in a range of hydrocarbon-contaminated environments [71-74]. Hydrocarbon-contaminated environments support the growth and development of microorganisms that preferentially utilize petroleum hydrocarbons as a carbon source. Similar to Sazykin et al. [72], our results demonstrated that *A. calcoaceticus* isolated from water (VKPM B-10353) and bottom sediment (EMBM-06) utilized hydrocarbons to create biosurfactants. This strain shares ancestry with a variety of *A. calcoaceticus* strains, as shown in Fig. 5, as indicated by phylogenetic analysis. These results are especially intriguing because previous research has indicated that *A. calcoaceticus* K-4 significantly inhibited the growth of *Candida albicans*, *Candida tropicalis, Candida utilis,* and *Saccharomyces cerevisiae* [75].

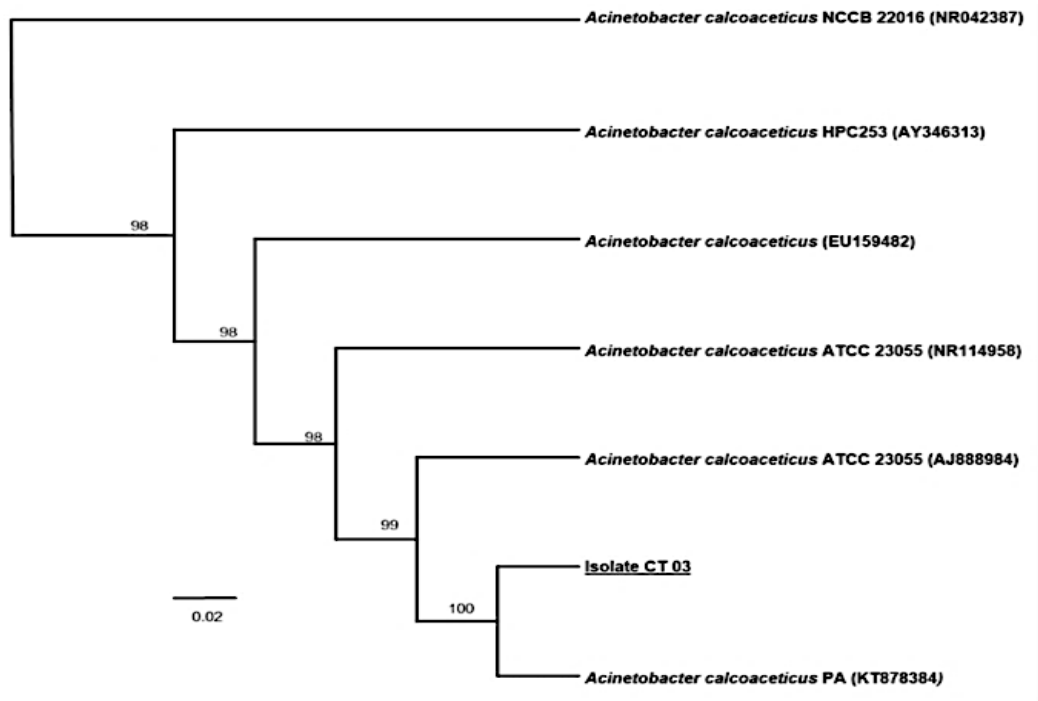

Fig. 5. The neighbor-joining approach of full *A. calcoaceticus* 16S rDNA sequences was used to generate a phylogenetic tree demonstrating the relative placements of antifungal isolate CT 03. The nodes of the tree display the bootstrap values resulting from a total of 100 repetition.

3.5 Factors influencing antifungal activity of biosurfactant-producing *A. calcoaceticus* CT 03

3.5.1 Carbon source and concentration

The dry cell weight, surface tension reduction, emulsification activity, and antifungal activity of *A. calcoaceticus* CT 03 were assessed in an MSM medium containing 1% of 9 carbon sources. The strain proved successful in proliferation and production of biosurfactant while using various carbon sources. The strain produced the largest inhibition area (10.74 mm) when glucose was used as the sole carbon source. The maximum levels of biomass production (2.44 g/L) and biosurfactant activity (23.50 mN/m and 25.50% for surface tension reduction and emulsification activity, respectively) were attained (Table 1). These results were in line with those of Wu et al. [76], who found that *Pseudomonas aeruginosa* EM1 isolated from an oil-contaminated area proliferated the fastest when glucose was used as the carbon source. As the glucose concentration grew, dry cell weight, emulsification activity, and antifungal activity increased, and surface tension decreased, with the highest output occurring at 4% (w/v) glucose (Table 3).

Table 1. Effect of various carbon sources on A. *calcoaceticus* CT 03 growth, biosurfactant generation, and antifungal activity when grown in 50 mL of media at 30 °C in a shaking incubator at 200 rpm for 48 hours.

C-source (1%)	Dry cell weight (*g/l*)*	Surface tension reduction (*mN/m*)*	Emulsification activity (*%*)*	Clear zone diameter (*mm*)*
No carbon source	0.08 ± 0.02^{f}**	2.00 ± 0.52^{c}**	0^{c}**	0^{c}**
Glucose	2.44 ± 1.03^{a}	23.50 ± 0.06^{a}	25.50 ± 0.25^{a}	10.74 ± 0.14^{a}
Commercial sucrose	2.25 ± 0.11^{b}	18.30 ± 0.16^{b}	15.12 ± 0.11^{c}	8.70 ± 0.28^{b}
Molasses	2.15 ± 0.02^{c}	10.80 ± 0.18^{c}	20.04 ± 0.07^{b}	7.02 ± 0.03^{b}
WPO	1.97 ± 0.19^{d}	8.50 ± 0.25^{d}	14.45 ± 0.21^{c}	5.04 ± 0.07^{d}
Soybean oil	1.84 ± 0.19^{d}	5.50 ± 0.22^{d}	10.05 ± 0.03^{d}	8.05 ± 0.02^{b}
Palm oil	1.50 ± 0.02^{e}	7.50 ± 0.15^{d}	28.54 ± 0.12^{a}	6.00 ± 0.02^{b}
Used palm oil	1.04 ± 0.19^{f}	6.01 ± 0.01^{d}	12.02 ± 0.02^{d}	5.00 ± 0.12^{d}

* Values are given as means ± SD from triplicate determinations.
** Different superscript letters in the same column indicate significant differences ($p < 0.05$).

Table 2. The effect of glucose concentration on *A. calcoaceticus* CT 03 growth, biosurfactant generation, and antifungal activity in a 250 ml flask containing 50 mL of glucose solution medium for 48 hours at 30 °C in a shaking incubator at 200 rpm.

Glucose (*%*)	Dry cell weight (*g/l*)*	Surface tension reduction (*mN/m*)*	Emulsification activity (*%*)*	Clear zone diameter (*mm*)*
0.5	1.26 ± 0.04^{d}**	15.10 ± 0.03^{d}**	12.51 ± 0.26^{c}**	5.25 ± 0.07^{c}**
1.0	2.45 ± 0.11^{c}	23.50 ± 0.05^{c}	25.50 ± 0.05^{d}	10.74 ± 0.30^{d}
2.0	2.71 ± 0.18^{b}	29.20 ± 0.01^{c}	31.25 ± 0.20^{c}	12.01 ± 0.04^{c}
3.0	2.95 ± 0.17^{b}	35.00 ± 0.12^{b}	42.03 ± 0.02^{b}	14.75 ± 0.14^{b}
4.0	3.14 ± 0.06^{a}	43.01 ± 0.02^{a}	50.15 ± 0.03^{a}	16.70 ± 0.15^{a}
5.0	3.28 ± 0.07^{a}	43.30 ± 0.02^{a}	50.05 ± 0.04^{a}	16.04 ± 0.01^{a}

* Values are given as means ± SD from triplicate determinations.
** Different superscript letters in the same column indicate significant differences ($p < 0.05$).

Table 3. Nitrogen source influence on growth, biosurfactant generation, and antifungal activity of *A. calcoaceticus* CT 03, grown in 50 mL nitrogen sources solution medium at 30 °C in a shaking incubator at 200 rpm for 48 hours.

Nitrogen source (*1%, w/v*)	Dry cell weight (*g/l*)*	Surface tension reduction (*mN/m*)*	Emulsification activity (*%*)*	Clear zone diameter (*mm*)*
No nitrogen source	0.06 ± 0.01^{d}**	1.50 ± 0.10^{f}**	0^{f}**	0^{d}**
Beef extract	3.05 ± 0.03^{b}	12.00 ± 0.01^{e}	25.50 ± 0.05^{e}	5.03 ± 0.04^{c}
CMSG	3.95 ± 0.17^{a}	19.00 ± 0.04^{d}	32.03 ± 0.09^{d}	10.70 ± 0.15^{b}
$NaNO_3$	2.54 ± 0.29^{c}	22.00 ± 0.02^{c}	36.08 ± 0.05^{c}	12.07 ± 0.03^{b}
$(NH4)_2SO_4$	3.14 ± 0.12^{b}	43.01 ± 0.02^{a}	50.15 ± 0.03^{a}	16.70 ± 0.19^{a}
NH_4Cl	2.44 ± 0.12^{c}	30.00 ± 0.02^{b}	40.15 ± 0.04^{b}	14.70 ± 0.18^{a}
NH_4NO_3	2.72 ± 0.04^{c}	25.00 ± 0.02^{c}	35.28 ± 0.07^{c}	12.01 ± 0.03^{b}
Peptone	4.11 ± 0.14^{a}	16.00 ± 0.04^{d}	30.01 ± 0.03^{d}	6.01 ± 0.03^{c}
Yeast extract	1.04 ± 0.03^{d}	18.00 ± 0.03^{d}	32.03 ± 0.14^{d}	5.86 ± 0.27^{c}

* Values are given as means ± SD from triplicate determinations.
** Different superscript letters in the same column indicate significant differences ($p < 0.05$).

3.5.2 Nitrogen source and concentration

The production of biosurfactant from *A. calcoaceticus* CT 03 was significantly impacted by a change in nitrogen source (Table 2). The strain produced the greatest biomass (4.11 g/L) in terms of dry cell weight when peptone was used as the only nitrogen source during cultivation. The largest inhibition area (16.60 mm) and maximum biosurfactant activity (43.1 mN/m and 50.15% for surface tension drop and emulsification activity, respectively) were found in the culture grown on $(NH_4)_2SO_4$ (Table 3). These results suggest that the best carbon source for biosurfactant synthesis is different from the best nitrogen source for *A. calcoaceticus* CT 03 growth. For the synthesis of biosurfactants, 1% (w/v) of $(NH_4)_2SO_4$ was the optimum concentration. Additionally, *A. calcoaceticus* CT 03 biosurfactant production was not significantly

affected by $(NH_4)_2SO_4$ concentration increases greater than 1% (w/v) (Table 4). The best performing growth medium, $(NH_4)_2SO_4$ at 1% (w/v), was selected for the subsequent biosurfactant production studies. Furthermore, Saimmai et al. [77] found that $(NH_4)_2SO_4$ boosted biosurfactant synthesis by *Leucobacter komagatae* 183 in comparison to other nitrogen sources.

Table 4. The effect of $(NH_4)_2SO_4$ concentration on *A. calcoaceticus* CT 03 growth, biosurfactant generation, and antifungal activity when grown in 50 mL of $(NH_4)_2SO_4$ solution medium at 30 °C in a shaking incubator at 200 rpm for 48 hours.

Nitrogen source (*1%, w/v*)	Dry cell weight (*g/l*)*	Surface tension reduction (*mN/m*)*	Emulsification activity (*%*)*	Clear zone diameter (*mm*)*
0.5	$2.86\pm0.27^{d**}$	$21.10\pm0.21^{c**}$	$35.51\pm0.25^{c**}$	$5.02\pm0.10^{c**}$
1.0	3.14 ± 0.10^{c}	43.80 ± 0.35^{a}	50.30 ± 0.52^{b}	16.70 ± 0.35^{b}
1.5	3.29 ± 0.05^{b}	43.00 ± 0.12^{a}	$50.15\pm.0.21^{b}$	16.15 ± 0.07^{b}
2.0	3.32 ± 0.20^{b}	42.30 ± 0.17^{b}	50.03 ± 0.09^{b}	16.70 ± 0.31^{b}
2.5	3.38 ± 0.14^{a}	42.80 ± 0.24^{b}	50.08 ± 0.11^{b}	17.70 ± 0.43^{a}
3.0	3.41 ± 0.17^{a}	43.50 ± 0.05^{a}	51.05 ± 0.04^{a}	16.70 ± 0.33^{b}

* Values are given as means ± SD from triplicate determinations.
** Different superscript letters in the same column indicate significant differences ($p < 0.05$)

3.5.3 Agitation

The results unambiguously demonstrated that agitation is required for *A. calcoaceticus* CT 03 to develop and produce biosurfactant because this organism cannot produce it in a stationary state. Agitation at a rate of 200 rpm resulted in the strain that produced the most biomass, reduced surface tension the most, emulsified the most, and inhibited the largest area. In the *A. calcoaceticus* CT 03 biosurfactant synthesis, agitation rates of more than 200 rpm had little impact (Table 5). The production of biosurfactants is triggered by agitation, according to other studies. One of the most important variables influencing *Natrialba* sp. M6's ability to produce biosurfactants, according to Hegazy et al. [78], is the agitation speed. *A. calcoaceticus* produced more biosurfactants when it was stirred at 200 rpm, as shown by Maqsood and Jamal [79]. High agitation rates, however, can obstruct oxygen transfer and limit the production of biosurfactants (Table 5).

Table 5. Effect of agitation on growth, biosurfactant generation, and antifungal activity of *A. calcoaceticus* CT 03 grown in MSM medium with 4% (w/v) glucose and 1% (w/v) $(NH_4)_2SO_4$ as carbon and nitrogen sources, respectively, for 48 hours at 30 °C in a shaking incubator.

Shaking speed (*rpm*)	Dry cell weight (*g/l*)*	Surface tension reduction (*mN/m*)*	Emulsification activity (*%*)*	Clear zone diameter (*mm*)*
50	$2.16\pm0.08^{d**}$	$17.10\pm0.05^{d**}$	$38.51\pm0.05^{d**}$	$12.51\pm0.30^{d**}$
100	2.85 ± 0.23^{c}	28.10 ± 0.07^{c}	42.51 ± 0.39^{c}	14.02 ± 0.07^{c}
150	3.00 ± 0.04^{b}	40.04 ± 0.45^{b}	47.08 ± 0.54^{b}	15.01 ± 0.12^{b}
200	3.14 ± 0.16^{b}	43.03 ± 0.20^{a}	50.15 ± 0.08^{a}	16.70 ± 0.40^{a}
250	3.24 ± 0.12^{a}	43.80 ± 0.35^{a}	50.15 ± 0.06^{a}	15.75 ± 0.24^{b}
300	3.48 ± 0.09^{a}	43.50 ± 0.35^{a}	50.05 ± 0.04^{a}	16.01 ± 0.03^{a}

* Values are given as means ± SD from triplicate determinations.
** Different superscript letters in the same column indicate significant differences ($p < 0.05$).

3.5.4 The medium pH

The rate of development and the production of biosurfactants are influenced by the growth medium's initial pH. The strain produced the most biomass (3.14 g/L), decreased surface tension the most (43.00 mN/m), had the highest emulsification activity (50.15%), and largest inhibition area (16.70 mm) when it was cultivated on culture medium with an initial pH of 7 (Table 6). It was discovered that the synthesis of biosurfactants decreased when the pH rose from 7.5 to 8.0.

Our findings are in line with earlier work by Saikia et al. [80]. According to Kumari et al. [81], the pH of the medium has a significant impact on the synthesis of biosurfactants in *Brevibacterium casei* LS14. Proper pH is essential for the growth and metabolism of the chosen strain. pH is maintained by metabolic processes that control the ion balances in cells [82, 83].

Table 6. The effects of initial pH on growth, biosurfactant production, and antifungal activity of *A. calcoaceticus* CT 03 in a 250-ml flask containing 4 % (w/v) glucose and 1 % (w/v) $(NH_4)_2SO_4$ as carbon and nitrogen sources, respectively, in MSM medium at 30 °C in a shaking incubator at 200 rpm for 48 hours.

Initial pH	Dry cell weight (*g/l*)*	Surface tension reduction (*mN/m*)*	Emulsification activity (*%*)*	Clear zone diameter (*mm*)*
5.0	1.96 ± 0.19^{d}**	20.50 ± 0.15^{e}**	20.25 ± 0.13^{e}**	4.51 ± 0.39^{f}**
5.5	2.15 ± 0.07^{c}	27.10 ± 0.07^{d}	28.51 ± 0.29^{d}	6.02 ± 0.07^{e}
6.0	2.82 ± 0.31^{b}	32.00 ± 0.09^{c}	37.03 ± 0.06^{c}	8.47 ± 0.42^{d}
6.5	3.04 ± 0.07^{a}	38.00 ± 0.03^{b}	44.07 ± 0.09^{b}	12.75 ± 0.30^{b}
7.0	3.14 ± 0.10^{a}	43.00 ± 0.04^{a}	50.15 ± 0.06^{a}	16.70 ± 0.35^{a}
7.5	3.01 ± 0.09^{a}	33.33 ± 0.22^{c}	43.05 ± 0.07^{b}	10.04 ± 0.05^{c}
8.0	2.82 ± 0.28^{b}	25.20 ± 0.23^{d}	31.52 ± 0.10^{d}	8.10 ± 0.13^{d}

* Values are given as means ± SD from triplicate determinations.
** Different superscript letters in the same column indicate significant differences ($p < 0.05$).

3.5.5 Times course

For 84 hours at 30 °C on a rotary shaker (200 rpm), *A. calcoaceticus* CT 03 was cultivated in a medium containing 4% (w/v) glucose and 1% (w/v) $(NH_4)_2SO_4$ as the carbon and nitrogen sources, respectively. This was done to promote the production of biosurfactants. Surface tension changes in the culture media show that *A. calcoaceticus* CT 03 started excreting biosurfactant after the lag phase, which was the first 9 hours of growth (Fig. 6). The highest biomass output (3.16 g/L) was noted when the culture had been in existence for 54 hours. The largest inhibition area (17.50 mm), lowest surface tension value (46.0 mN/m), and highest emulsification activity (54.0%) were noted after 60 hours of culture. In order to develop biosurfactants, strain *A. calcoaceticus* CT 03 was cultured for 60 hours. The findings support the claim by Suwansukho et al. [84], that *Bacillus subtilis* MUV4 releases biosurfactants as secondary metabolites. These results demonstrated that biosurfactants were generated by *A. calcoaceticus* CT 03 in response to growth [85].

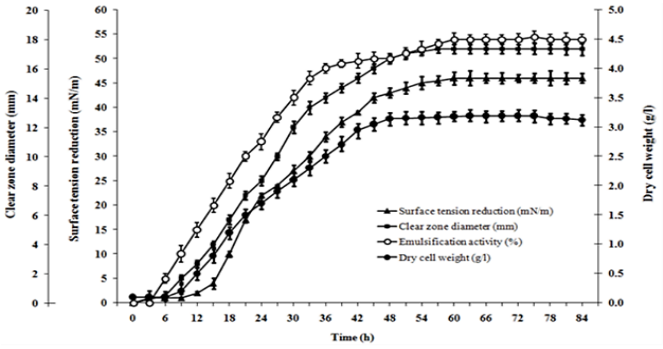

Fig. 6. Time course of *A. calcoaceticus* CT 03 biosurfactant synthesis in molasses medium containing 4% (w/v) glucose and 1% (w/v) $(NH_4)_2SO_4$. The standard deviation from three determinations is represented by the bars.

3.6 Recovery of Biosurfactant

The biosurfactant was precipitated or isolated from the culture supernatant of *A. calcoaceticus* CT 03 strain. The supernatant of this culture was most successfully precipitated and extracted using a chloroform methanol solution at a 2: 1 ratio (Table 7). A recovery yield of 0.175 g/L (dry weight) was recorded for *A. calcoaceticus* CT 03. It was calculated that under these circumstances, the specific yield for the production of crude biosurfactants was 0.438 $kg_{product}/kg_{substrate}$. Previous research studies [86-92] have observed low biosurfactant specific yield in the range of 0.0075 to 0.194 $kg_{product}/kg_{substrate}$ when glucose was utilized as a substrate. Almansoory et al. [93] showed that *P. fluorescens* produced a greater biosurfactant specific yield (2.0 $kg_{product}/kg_{substrate}$) when NH_4NO_3 was used as a substrate.

Table 7. Effect of recovery method on yield, EA and EI of the biosurfactant produced by *A. calcoaceticus* CT 03.

Recovery method	Yield, g/L (*g/l*)*	Emulsification Activity (%)*	Emulsification Index (%)*
Acid precipitation	5.62 ± 2.15a**	43.12 ± 2.35a**	33.25 ± 3.20a**
Acetone precipitation	5.08 ± 1.8a	52.14 ± 2.14a	43.12 ± 3.48a
$(NH_4)_2SO_4$ precipitation	4.26 ± 0.54a	53.62 ± 3.52a	48.94 ± 2.69a
MeOH precipitation	2.28 ± 0.39b	61.37 ± 4.25b	52.64 ± 2.34b
EtOH precipitation	1.96 ± 0.72b	69.48 ± 4.68b	49.55 ± 3.45b
CH_3Cl : MeOH extraction	1.75 ± 0.28b	78.26 ± 2.42b	58.75 ± 3.21b

* Values are given as means ± SD from triplicate determinations.

** Different superscript letters in the same column indicate significant differences ($p < 0.05$).

3.7 Biosurfactant Characteristics

The lipopeptide structure of biosurfactant from *A. calcoaceticus* CT 03 was revealed by the FTIR adsorption spectra to contain peptide, aliphatic, and carbonyl functional groups (Fig. 7). The largest adsorption bands at 3421 and 3308 cm^{-1} were assigned to the -NH and -OH lengths of the peptide. In contrast to the 1550 cm^{-1} band, where the N-H bond and the stretching of the CO-N bond were combined, the characteristic absorbance at band 1652 cm^{-1} predicted the stretching of the CO-N bond. Absorption peaks at 1652 and 1550 cm^{-1} proved the existence of a carbonyl (-C=O) group. Significant absorbance peaks at 1403, 1467, 2855, and 2928 cm^{-1} [94] demonstrated the existence of a biosurfactant with an aliphatic long-chain hydrocarbon. These findings supported the biosurfactant's lipopeptide nature [95-98].

To validate the results of this inquiry, an NMR analysis was conducted (Fig. 8a). The molecule was determined to be a lipopeptide, according to the 1H-NMR data. The majority of the backbone amide NH groups are located between 8.4 and 7.2 ppm downfield from tetramethylsilane (Fig. 8b). The amino acid's alpha hydrogens resonate with the peptide moiety between 5.0 and 3.8 ppm. The fatty acid component may have terminal branching, according to a doublet for the $(CH_3)_2$-CH group at less than 0.90 ppm. Because CH was present at 1.3 ppm, it was unable to estimate the ratio of the terminal and methylene groups. The sidechain protons of the amino acids led to the formation of additional multiplets in the upfield region. The remaining spectra clearly demonstrated the existence of -hydroxy fatty acid. The discovered biosurfactant shares similarities with surfactin, a member of the lipopeptide-type biosurfactant family, in its NMR spectrogram presence [99-101].

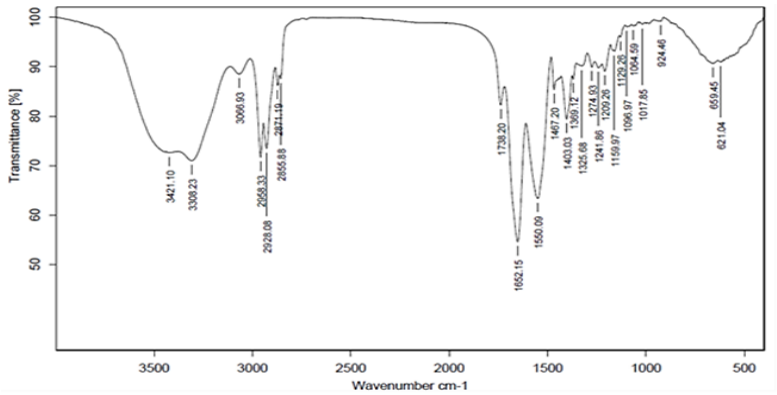

Fig. 7. FT-IR spectrum of the biosurfactant produced by *A. calcoaceticu*.

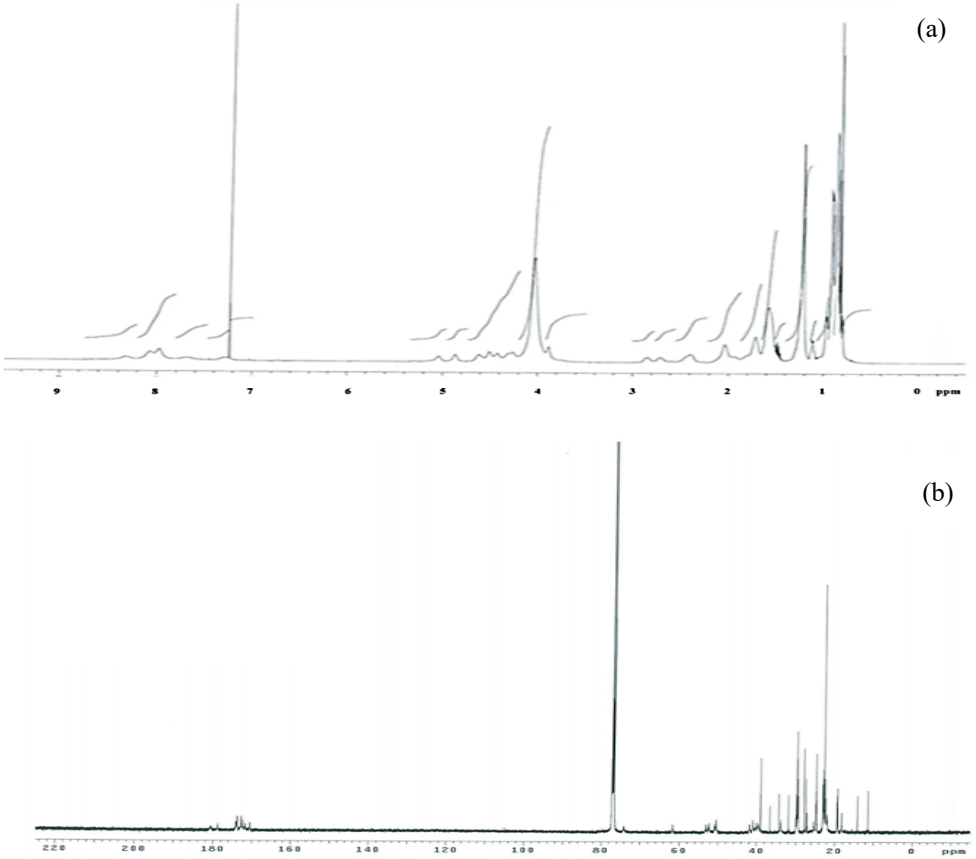

Fig. 8. ^{1}H-NMR spectrum (a) and ^{13}C-NMR (b) NMR spectra of the biosurfactant produced by *A. calcoaceticus* CT 03.

4. Conclusions

A strain of *A. calcoaceticus* from oil-polluted soil strain in southern Thailand designated CT 03, produces biosurfactants that significantly inhibited the growth of *P. palmivora*, the organism responsible for black rot disease of orchids. Results suggest that the qualities of the biosurfactant may

have potential industrial applications. Lipopeptide was found in the molecules of the biosurfactant after being chemically characterized using FT-IR and NMR spectroscopy. Importantly, the use of biosurfactants as antifungal agents has real-world applications. The results of this study will help reduce the use of harsh synthetic biosurfactants and fungicides, benefiting the environment and protecting orchids from disease.

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