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Identification and Characterization of the Yellow Pigment Synthesized by *Cupriavidus* sp. USMAHM13

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Abstract Microbial pigments are gaining intensive attention due to increasing awareness of the toxicity of synthetic colours. In this study, a novel polymer-producing bacterium designated as *Cupriavidus* sp. USMAHM13 was also found to produce yellow pigment when cultivated in nutrient broth. Various parameters such as temperature, pH and ratio of culture volume to flask volume were found to influence the yellow pigment production. UV-Visible, Fourier transform infrared and ^{13}C-nuclear magnetic resonance analyses revealed that the crude yellow pigment might probably represent new bioactive compound in the carotenoid family. The crude yellow pigment also exhibited a wide spectrum of antimicrobial activity against Gram-negative and Gram-positive bacteria with their inhibition zones and minimal inhibitory concentrations ranged from 25 to 38 mm and from 0.63 to 2.5 mg/ml, respectively. To the best of our knowledge, this is the first report on the identification and characterization of yellow pigment produced by bacterium belonging to the genus *Cupriavidus*.

Keywords Bioactive compound · Carotenoids · *Cupriavidus* sp. · Microbial pigment · Natural pigment · Antimicrobial activity

Introduction

Microbial pigments are referred to the colour produced by the microorganisms. They are in increasing demand although variety of the pigments from fruits and vegetables is available in

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abundance because their production is not dependent on the seasons and geographical conditions. The microbial pigments are also considered natural and safe to use in various industrial applications. Controllable and predictable yield of pigment can also be attained by using pigment-producing microorganism. Microbial pigments also have more unique and attractive properties such as colours of different shades which are not found in plant. They also provide nutrients like vitamins and exhibit antimicrobial and medicinal properties. Water and environmental pollution can be reduced too as some of the pigments are produced by the microorganisms using industrial residues such as starch and juice industry [1]. Ability to synthesize pigments through easier and faster growth in the cheap culture medium is one of the advantages of pigment production from microorganisms [2].

The potential pigment-producing microorganisms should satisfy few requirements such as ability to use various carbon and nitrogen sources with moderate growth conditions and reasonable colour yield. A good pigment producer should also be able to tolerate wide range of pH, temperature and mineral concentration. Most important is it should be non-toxic, non-pathogenic and must be easily recovered from the cells [3].

Carotenoids are one of the widely distributed classes of pigments in nature, and their colours range from light yellow through orange to deep red. More than 600 carotenoids have been discovered and characterized structurally. Carotenoids are found to exhibit anticancer, antibiotic and immunosuppressive activities. Generally, these pigments can repress or kill other microorganisms at very low concentration. They offer imperative role in both agricultural and pharmaceutical research considering their diverse and potential activities against different types of diseases. They also have functions as food colourants, nutritional supplements, feed additives to enhance the pigmentation of fish and eggs [4, 5] and as structural component of microbial membranes [6].

The present study was focused on investigating the factors influencing bacterial growth and yellow pigment production by *Cupriavidus* sp. USMAHM13 in nutrient-rich broth medium. The crude yellow pigment was extracted by solvent extraction method using methanol and identified based on UV-vis absorption spectrum, ^{13}C-nuclear magnetic resonance (^{13}C-NMR) and Fourier transform infrared (FT-IR) analysis. The antimicrobial activity of the crude yellow pigment extract against the microorganisms was also assayed through the disc diffusion method and minimal inhibitory concentration determination.

Materials and Methods

Microorganism and Culture Medium

The strain used in this study was isolated from paddy field that is located in Perak, Malaysia. It was deposited in the German Culture Collection of Microorganisms and Cell Cultures (DSM 25816) and in the Korean Collection for Type Cultures (KCTC 32390) as *Cupriavidus* sp. USMAHM13. The GenBank accession number for the 16S rRNA gene sequences (Table 1) of the strain *Cupriavidus* sp. USMAHM13 is KF460028. Nutrient-rich medium composed of (g/l) meat extract (10), peptone (10) and yeast extract (2) was used for bacterial growth of *Cupriavidus* sp. USMAHM13.

Effect of Various Parameters on the Yellow Pigment Production

Effect of temperature (from 20 to 55 °C), pH (from 4 to 11) and ratio of culture volume to flask volume (from 30/250 to 80/250) on the yellow pigment production was also studied by

Table 1 Complete 16S rRNA gene sequence of the *Cupriavidus* sp. USMAHM13

TTTGATCCTG	GCTCAGATTG	AACGCTGGCG	GCATGCCTTA	CACATGCAAG
TCGAACGGCA	GCGCGGACTT	CGGTCTGGCG	GCGAGTGGCG	AACGGGTGAG
TAATACATCG	GAACGTGCCC	TGTTGTGGGG	GATAACTAGT	CGAAAGATTA
GCTAATACCG	CATACGACCT	GAGGGTGAAA	GCGGGGGACC	GYAAGGCCTC
GCGCAATAGG	AGCGGCCGAT	GTCTGATTAG	CTAGTTGGTG	AGGTAAAGGC
TCACCAAGGC	GACGATCAGT	AGCTGGTCTG	AGAGGACGAT	CAGCCACACT
GGGACTGAGA	CACGGCCCAG	ACTCCTACGG	GAGGCAGCAG	TGGGGAATTT
TGGACAATGG	GGGCAACCCT	GATCCAGCAA	TGCCGCGTGT	GTGAAGAAGG
CCTTCGGGTT	GTAAAGCACT	TTTGTCCGGA	AAGAAATCCT	TTGGGCTAAT
ACCCCGGAGG	GATGACGGTA	CCGGAAGAAT	AAGCACCGGC	TAACTACGTG
CCAGCAGCCG	CGGTAATACA	TAGGGTGCGA	GCGTTAATCG	GAATTACTGG
GCGTAAAGCG	TGCGCAGGCG	GTTTTGTAAG	ACAGGCGTGA	AATCCCCGGG
CTCAACCTGG	GAATGGCGCT	TGTGACTGCA	AGGCTAGAGT	GCGTCAGAGG
GGGGTAGAAT	TCCACGTGTA	GCAGTGAAAT	GCGTAGAGAT	GTGGAGGAAT
ACCGATGGCG	AAGGCAGCCC	CCTGGGACGT	GACTGACGCT	CATGCACGAA
AGCGTGGGGA	GCAAACAGGA	TTAGATACCC	TGGTAGTCCA	CGCCCTAAAC
GATGTCAACT	AGTTGTTGGG	GATTCATTTC	TTCAGTAACG	AAGCTAACGC
GTGAAGTTGA	CCGCCTGGGG	AGTACGGTCG	CAAGATTAAA	ACTCAAAGGA
ATTGACGGGG	ACCCGCACAA	GCGGTGGATG	ATGTGGATTA	ATTCGATGCA
ACGCGAAAAA	CCTTACCTAC	CCTTGACATG	CCACTAACGA	AGCAGAGATG
CATTAGGTGC	CCGAAAGGGA	AAGTGGACAC	AGGTGCTGCA	TGGCTGTCGT
CAGCTCGTGT	CGTGAGATGT	TGGGTTAAGT	CCCGCAACGA	GCGCAACCCT
TGTCTCTAGT	TGCTACGAAA	GGGCACTCTA	GAGAGACTGC	CGGTGACAAA
CCGGAGGAAG	GTGGGGATGA	CGTCAAGTCC	TCATGGCCCT	TATGGGTAGG
GCTTCACACG	TCATACAATG	GTGCGTACAG	AGGGTTGCCA	ACCCGCGAGG
GGGAGCTAAT	CCCAGAAAAC	GCATCGTAGT	CCGGATCGTA	GTCTGCAACT
CGACTACGTG	AAGCTGGAAT	CGCTAGTAAT	CGCGGATCAG	CATGCCGCGG
TGAATACGTT	CCCGGGTCTT	GTACACACCG	CCCGTCACAC	CATGGGAGTG
GGTTTTGCCA	GAAGTAGTTA	GCCTAACCGC	AAGGAGGGCG	ATTACCACGG
CAGGGTTCAT	GACTGGGGTG	AAGTCGTAAC	AAGGTAGCCG	TACCGGAAGG
TGCGGCTGGA	TCACCTCCTT			
TC				

The 16S rRNA gene sequence was determined by Leibniz-Institute DSMZ-German Collection of Microorganisms and Cell Cultures. The sequence consisted of 1,522 bp (base count 384 A, 352 C, 485 G, 300 T and 1 others)

transferring pre-cultured cells (0.05 g/l) of *Cupriavidus* sp. USMAHM13 into 50 ml of NR broth. The bacterial cultures were incubated for 24 h at 200 rpm in the orbital shaker. All experiments were carried out in triplicates and results documented as the average of three independent measurements. Bacterial growth and pigmentation were determined in terms of optical density according to Ramachandran and Amirul [7].

Identification of the Crude Yellow Pigment

The crude yellow pigment was extracted from the freeze-dried cells of *Cupriavidus* sp. USMAHM13 and identified based on the absorption spectra by scanning the absorbance in

the wavelength region of 380–600 nm using the UV-vis spectrophotometer. Thin film method was employed to analyse the pigment using FT-IR spectrophotometer (FTIR-2000, Perkin Elmer, USA). The yellow pigment (10 mg) was dissolved in chloroform and transferred onto thallium bromide disc. Once the chloroform evaporated, the disc was exposed to IR radiations in the range of 400–4,000 cm^{-1}. The yellow pigment (10 mg) was also dissolved in deuterated chloroform ($CDCl_3$) and analysed using ^{13}C-NMR at 400 MHz (Bruker, Switzerland) at 25 °C. Chemical shifts (ppm) in NMR analysis were reported relative to tetramethylsilane (TMS).

Antimicrobial Activity Assay of the Crude Yellow Pigment

Disc Diffusion Assay

Suspension of bacteria was spread evenly on the NA. The discs (6 mm in diameter) were individually impregnated with 60 μl of the crude pigment (20 mg/ml) and placed onto the agar plates which had been previously inoculated with the tested bacteria: *Bacillus thuringiensis* ATCC 10792, *Pseudomonas aeruginosa* USM-AR2 (local isolate), *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 11303 and *Staphylococcus aureus* ATCC 12600. The plates were incubated at 37 °C for 24 h. Methanol was used as control in this assay.

Minimal Inhibitory Concentration Determination

The crude pigment extract was first diluted to the highest concentration (20 mg/ml). About 95 μl of nutrient broth (NB) containing per litre of peptone, 5 g; yeast extract, 2 g: lab-lemco, 1 g and NaCl, 5 g was added to each tube followed by the addition of 100 μl of pigment extract, and then, serial dilutions were performed consecutively in concentration range from 10 to 0.63 mg/ml with NB. Tested microorganisms (5 μl) grown for 18 h in NB were cultured into each tube. Tested microorganisms were *P. aeruginosa* USM-AR2 (local isolate), *B. subtilis* ATCC 6633, *E. coli* ATCC 11303 and *S. aureus* ATCC 12600. After incubating the cell cultures for 24 h, serial dilution was performed and 10 μl of bacterial cultures from each tube was spread evenly onto NA.

Results and Discussion

Factors Influencing Yellow Pigment Production by *Cupriavidus* sp. USMAHM13

Previous study revealed that *Cupriavidus* sp. USMAHM13 capable of producing yellow pigment on nutrient agar and the pigmentation of the strain USMAHM13 exhibited a growth-associated mode when grown in nutrient-rich broth medium [7]. Further studies were performed in this study to characterize and identify the crude yellow pigment. Figure 1 demonstrates the effect of temperature, pH and ratio of culture volume to flask volume (aeration) on the bacterial growth and yellow pigment production by *Cupriavidus* sp. USMAHM13. It was evident that maximum bacterial growth and pigment production were obtained at 28 °C which clearly indicated the mesophilic nature of the *Cupriavidus* sp. USMAHM13 (Fig. 1a). Although, bacterial growth was detected at temperature of 37 °C, but yellow pigment was not synthesized which indicated temperature dependence of the yellow pigment production by *Cupriavidus* sp. USMAHM13. At temperature beyond 45 °C, bacterial growth and pigmentation were not observed. Similar observation was reported by Venil and Lakshmanaperumalsamy [2] whereby maximum prodigiosin production by *Serratia*

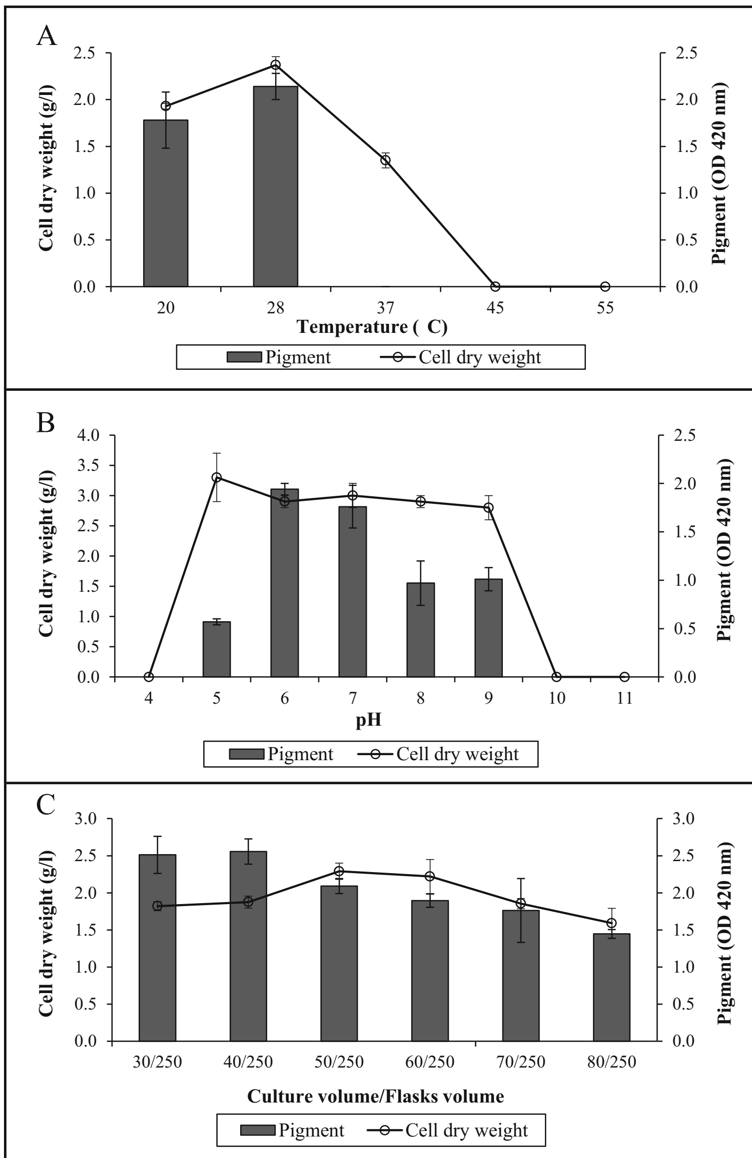

Fig. 1 Effect of **a** temperature, **b** pH and **c** culture volume/flask volume on the bacterial growth and yellow pigment production by *Cupriavidus* sp. USMAHM13. Cells were cultivated in growth medium (NR)

marcescens was seen at 28 and 30 °C in nutrient broth. At 37 °C, *S. marcescens* did not show any pigment production in nutrient broth and the culture broth was white in colour.

It was observed that at pH of 4, 10 and 11, there was no bacterial growth and pigment production by *Cupriavidus* sp. USMAHM13 (Fig. 1b). Highly acidic or alkaline pH would inhibit the cells and decrease the pigment production as they could denature the intracellular enzymes or alter the cell structure. Almost all carotenoids would disintegrate, dehydrate or isomerize in acidic condition, especially in the presence of light [8]. The bacterium has ability

to grow at pH ranging from 5 to 9; however, the pigment production was observed significantly high only at pH 6 and 7, suggesting no direct correlation between bacterial growth and pigment production at different pHs.

Aerating the growth medium was extremely important for the optimum bacterial growth and production of pigment because *Cupriavidus* sp. USMAHM13 is an aerobic microorganism, and therefore, it required the provision of oxygen. Higher pigment production was observed at lower culture volume of 30 and 40 ml (Fig. 1c). High dissolved oxygen transfer (DOT) is obtained when the culture volume is approximately 10–25 % of the flask volume. This is because a smaller volume will maximize the surface area exposed to the air. It could be suggested that the pigment was enhanced at high aeration condition in order to protect cells from oxidative damage that resulted from excess of oxygen in the culture broth. It had been reported that reactive oxygen species (ROS) which increased with exposure to air containing oxygen could induce carotenogenesis. Carotenoid has also an important role as an anti-oxidative reagent. Consequently, carotenoid must be synthesized to eliminate intracellular ROS rapidly [9]. Then, the pigment production gradually decreased with the increase of culture volume. However, the bacterial growth did not show significant differences at different ratios of culture volume to flask volume.

Identification of the Crude Yellow Pigment

The colour of the pigment provides the first clues for the identification of the crude yellow pigment. The pigment extracted from the *Cupriavidus* sp. USMAHM13 exhibited variation in the colour from bright yellow to deep red. According to Namitha and Negi [4], the colour of the carotenoids also ranges from light yellow through orange to deep red. The crude yellow pigment as characterized based on the absorption spectrum showed that it exhibited maximum absorption at 420 nm [7], suggesting that the pigment is a possible carotenoid because the carotenoids have a maximum absorption at a wavelength of 300–600 nm [5].

The FT-IR spectrum of the extracted yellow pigment showed the presence of carbonyl functional group by indicating a sharp peak at 1,729 cm^{-1} (Fig. 2). This carbonyl group might

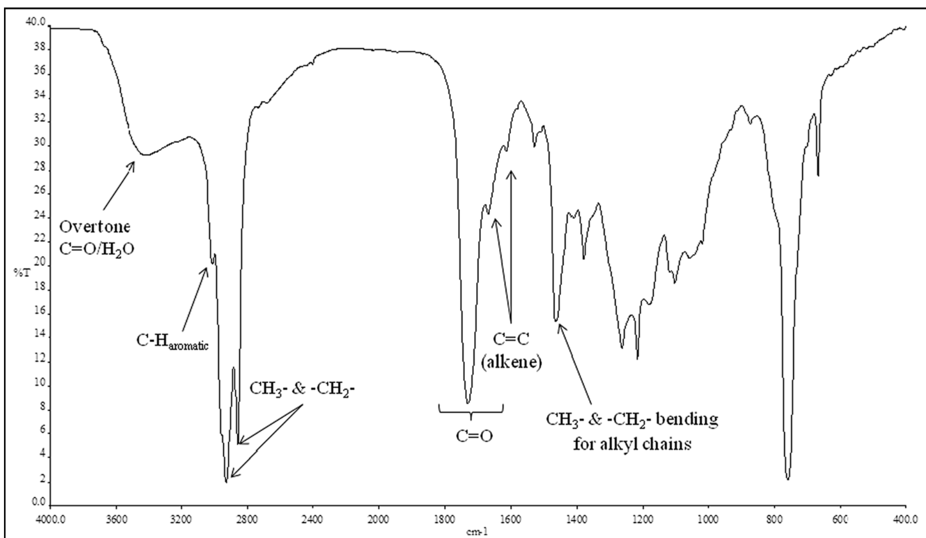

Fig. 2 FT-IR spectrum of the crude yellow pigment synthesized by *Cupriavidus* sp. USMAHM13

belong either to aldehyde, ester or carboxylic families. An observation of a weak and broad signal at around 3,435 cm^{-1} provided further evidence that a carboxylic family is present in the compound. The presence of carboxylic family was further supported by ^{13}C-NMR analysis (Fig. 3) whereby two signals were observed at δ 172.9 and 176.8 ppm. This region (δ 160–180 ppm) is specific for carboxylic acids and esters. Saturated esters showed carbonyl peaks around 1,735 cm^{-1}, and possibility exists for its presence as the spectrum also has some additional vibrational bands in the range of 1,100–1,350 cm^{-1} that appeared for (C-O-R) stretch. The possibility of presence of esteric group also increased due to appearance of carbonyl signals in the range of δ 160–180 ppm of ^{13}C-NMR spectrum.

The possibility of aldehyde group was ignored due to the absence of two bands for aldehyde C-H which are usually observed at 2,700–2,760 cm^{-1} and 2,800–2,860 cm^{-1}. This was further affirmed by the absence of aldehyde carbonyl peak in the region δ 180–210 ppm of the ^{13}C-NMR spectrum. The possibilities of ketonic and other carbonyl containing compounds (acid halides and amides) were not possible too because the vibrational band which appeared at 1,729 cm^{-1} did not favour the presence of these carbonyls. Absence of ketonic carbonyl peaks in the range of δ 180–220^{+} ppm of the ^{13}C-NMR spectrum further confirmed this observation. Furthermore, two adjacent and sharp vibrational bands at 2,924 and 2,854 cm^{-1} indicated the presence of alkyl chains which was further confirmed by ^{13}C-NMR spectrum. The presence of alkenes (C=C) was supported by a strong vibrational band at 1,666 and 1,610 cm^{-1}.

Majority of carotenoids are derived from a 40-carbon polyene chain which could be considered as the backbone of the molecule and divided into two groups (carotene and xanthophylls). Carotenes are purely hydrocarbons whereas xanthophylls are the oxygenated derivatives of the hydrocarbons [4]. Based on the FT-IR and ^{13}C-NMR spectrum, it could be suggested that the yellow pigment might belong to the xanthophylls group as the spectrum

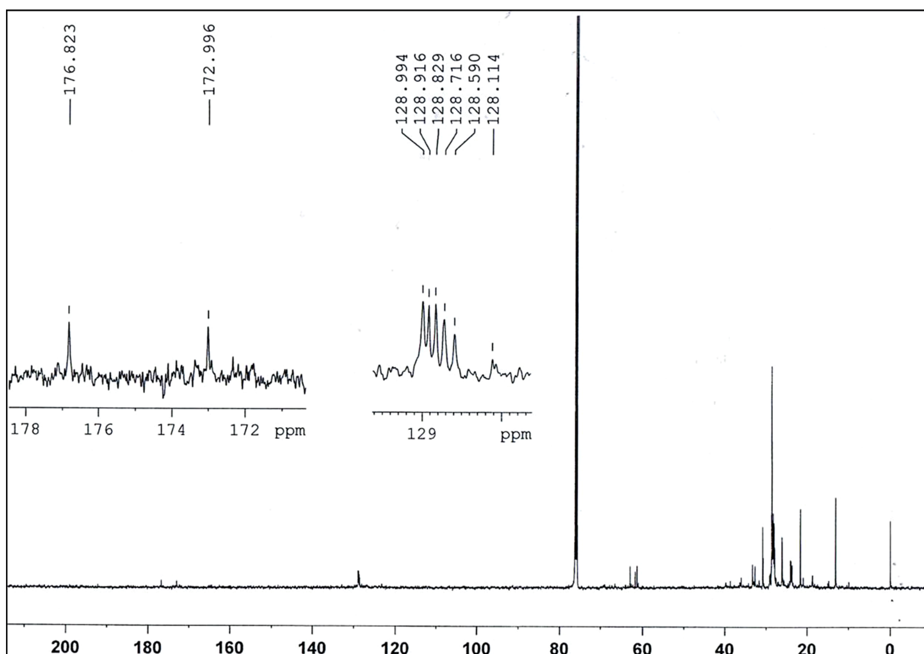

Fig. 3 ^{13}C-NMR spectrum of the crude yellow pigment synthesized by *Cupriavidus* sp. USMAHM13

revealed the presence of carboxylic acid and ester group. FT-IR and ^{13}C-NMR spectrum also showed the existence of longer saturated and unsaturated hydrocarbon chains as backbone which further affirmed the resemblance of yellow pigment to the carotenoids.

Antimicrobial Properties of the Crude Yellow Pigment

The antimicrobial activity of the crude yellow pigment extract of *Cupriavidus* sp. USMAHM13 against the microorganisms was qualitatively and quantitatively assayed through the disc diffusion method (Fig. 4) and minimal inhibitory concentration (MIC) (Table 2). The data obtained from the disc diffusion method indicated that the pigment extract exhibited good antibacterial properties but displayed a variable degree of antimicrobial activity against different tested strains. Its inhibitory effect on the Gram-positive bacteria (*S. aureus* ATCC 12600, *B. thuringiensis* ATCC 10792 and *B. subtilis* ATCC 6633) was greater than Gram-negative bacteria (*P. aeruginosa* USM-AR2 and *E. coli* ATCC 11303). The most susceptible bacterium to yellow pigment extract was *B. subtilis* with the highest inhibition zone of 38 mm followed by *B. thuringiensis* ATCC 10792 (30 mm) and *S. aureus* ATCC 12600 (29 mm). The yellow pigment extract showed moderate activity to *E. coli* and *P. aeruginosa*, evidenced by the inhibition zone of 25 and 26 mm, respectively.

The data for MIC also showed that the crude yellow pigment extract exhibited varying levels of antimicrobial activity against the tested microorganisms. The minimal inhibitory

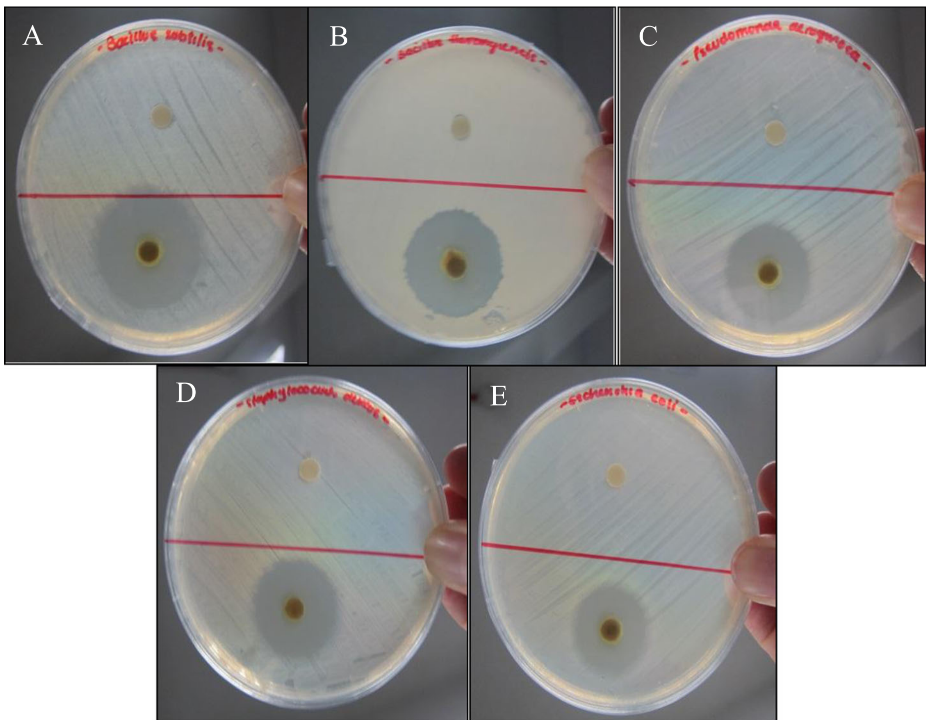

Fig. 4 Antimicrobial assay of the crude yellow pigment synthesized by *Cupriavidus* sp. USMAHM13 against five different microorganisms. **a** *Bacillus subtilis* ATCC 6633, **b** *Bacillus thuringiensis* ATCC 10792, **c** *Pseudomonas aeruginosa* USM-AR2 (local isolate), **d** *Staphylococcus aureus* ATCC 12600 and **e** *Escherichia coli* ATCC 11303. Methanol was used as control

Table 2 Minimal inhibitory concentration of the crude yellow pigment extracted from *Cupriavidus* sp. USMAHM13 against four different microorganisms

Tested microorganisms	MIC value (mg/ml)
Escherichia coli ATCC 11303	2.50
Pseudomonas aeruginosa USM-AR2	1.25
Staphylococcus aureus ATCC 12600	1.25
Bacillus subtilis ATCC 6633	0.63

concentrations were observed within a range of 0.63 to 2.5 mg/ml. Maximum activity was observed against *B. subtilis* with MIC value of 0.63 mg/ml. *S. aureus* and *P. aeruginosa* showed similar susceptibility to the yellow pigment extract with MIC value of 1.25 mg/ml. Weak inhibitory effect of yellow pigment extract was observed against *E. coli* with MIC value of 2.50 mg/ml. These results were in accordance with those obtained using disc diffusion assay.

Pseudoalteromonas sp. H1.7, a marine bacterium associated with algae *Halimeda* sp., produced yellow pigments which were found to belong to the group of carotenoid, especially the member of xanthophylls. The yellow pigment exhibited strong growth inhibition against *S. aureus* with inhibition zone of 29 mm [10]. The antimicrobial action of violet pigment, a mixture of violacein and deoxyviolacein, isolated from phychrotrophic bacterium RT102 strain was confirmed for several bacteria such as *Bacillus licheniformis*, *B. subtilis*, *Bacillus megaterium*, *S. aureus* and *P. aeruginosa*, and the high concentration of violet pigment (>15 mg/l) caused not only growth inhibition but also death of cells. However, it could not inhibit the growth of Gram-negative bacteria such as *Flavobacterium balustinum* and *E. coli* even if more than 50 mg/l violet pigment was added [11].

Conclusion

The pigment extracted from *Cupriavidus* sp. USMAHM13 might probably represent a novel compound in the carotenoid group as no reports available for the production of carotenoid by bacteria belong to the genus *Cupriavidus*. The FT-IR and ^{13}C-NMR spectrum also revealed that the yellow pigment might consist of more than one compounds. Therefore, further purification and characterization are required to elucidate the structure of this yellow pigment. The role of yellow pigment in life and metabolism of *Cupriavidus* sp. USMAHM13 is at present not fully understood. It could be assumed that the antimicrobial property of the yellow pigment might play a very important role in the survival and diffusion of this bacterium in hostile environments. However, the metabolic pathway by which the survival is guaranteed is not understood and further studies are needed to deepen these aspects.

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