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RESEARCH ARTICLE

Influence of growth factors on pigmentation of *Chaetomium cupreum* SS – 02 and the antibacterial efficacy of the pigment against *Ralstonia solanacearum*

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Abstract

An extracellular pigment-producing ascomycetous filamentous fungus *Chaetomium* was isolated from litter sample and identified as *Chaetomium cupreum* SS-02. The optimal growth conditions for high pigment yield and the *invitro* antagonistic activity of the pigment against *Ralstonia solanacearum* – a phytopathogenic bacterium, was investigated. The optimal culture conditions for pigment production were as follows; Dextrose 2%, Peptone + Yeast extract (0.4%), pH 6, temperature 35°C and inoculum age - 6 days old. The *invitro* screening of antibacterial activity of the pigment against five pathogenic strains of *Ralstonia solanacearum* was measured in terms of zone of inhibition. The highest activity was recorded against RS4 strain.

Introduction

Color, in one form or the other has been a vital part of many industries from centuries together. The existing authorized natural colorants are either produced from plant or animal origin and have various drawbacks such as instability against light, heat or pH, low solubility in water, and are often not available throughout the year (Sameer et al., 2006). The demand for such natural compounds is increasing steeply due to awareness of positive health benefits out of such compounds (PrithamChattopadhyay et al., 2008). Interest in microbial pigments is ever-increasing and insatiable owing to their nature, medicinal properties, nutritive value, expected yield, easy handling, safety, production being independent of season and geographical conditions (Latha et al., 2010).

There are a good number of micro-organisms which are able to produce pigments in high yields. Those organisms include species of *Monascus, Paecilomyces, Serratia, Cordyceps, Streptomyces* and yellow-red and blue compounds produced by *Penicillium herquei* and *Penicillium atrovenetum.* Among them, many fungi are potentially promising because of their capability of producing different coloured pigments with high chemical stability (Hajjaj et al., 2000). For industrial applications, microbial pigments should be produced in high yield with chemical and light stability.

Chaetomium cupreum is an abundantly found soil fungus exhibiting antagonism against numerous fungal phytopathogens(Mao et al., 2010). Ketomium®, a commercial product, has been developed from this species, and is being widely used as broad spectrum bio- fungicide for the disease control in various crops (Soytong et al., 2001). It is also known to biodegrade catechin, a well-known recalcitrant compound (Arunachalam et al., 2003). Azaphilones, a novel bicyclic anthraquinone has been successfully isolated from it and a red colored compound, oosporein isolated from it is known to have antifungal effects against *Rhizoctonia solani, Botrytis cinerea, Pytium ultimum* and many pathogenic fungi. It is also known to have antitumor activity against HL-60 and A549 and acute toxicity against *Artemia salina*(Kanokmedhakul et al., 2007). Its antifungal activity is being exploited for natural medicines (Kanokmedhakul et al., 2006)andit is known to inhibit *Candida albicans* (Vengurlekaret al., 2012).The halogenated compound rubrorotiorin, is reported to be the most active compound (Saleemet al., 2010).

Though *C. cupreum* has been widely used as biocontrol agent against fungal phytopathogens, its antagonistic activity against phytopathogenic bacteria is not well established. In this study we have screened the pigment extract for its efficacy to inhibit and control the growth of *Ralstonia solanacearum*, a bacterial

phytopathogen causing wilt of tomato plants (Economically important crop grown worldwide). This organism causes yield loss upto 90% depending on the stage of the plant growth at the time of infection. (Singh, 2005; Kumar et al., 2002).

Our attention was focused to isolate and optimize the fermentation conditions in order to produce higher yield of the pigment from *C. cupreum*, which is necessary for commercial applications and to evaluate the antibacterial activity of the pigment extract against *R. solanacearum.*

Materials and Methods

Microorganism and inoculum preparation:

Chaetomium cupreum SS-02 was isolated from litter sample and was identified by morphological and molecular methods. The morphological identity was confirmed by National Fungal Culture Collection of India (NFCCI), Agharkar Research Institute, Pune, India. To confirm the species, sequence analysis of the Internal Transcribed Spacer (ITS) region using universal primers (ITS 1 and ITS 4) was performed. Nucleotide BLAST to the obtained sequence was performed in National Center for Biotechnology Information (NCBI - www.ncbi.nlm.nih.gov/) using "blastn" suite. The sequence was deposited in NCBI Genbank.

The stock culture was maintained on a potato dextrose agar (PDA) slant. For inoculum preparation, the fungus was grown at 25°C on a PDA plate for 5 days. 0.5 cm^2 mycelial discs bored out from the periphery of the colony transferred to 100 ml of the optimization media and incubated at 26 ± 2°C on a rotary shaker at 120 rpm for 5 days. (Gunasekaran et al., 2008).

Optimization of culture conditions:

The total pigmentation and biomass yield of *C. cupreum* SS-02 were studied in a culture medium containing (g L^{-1}) glucose (C Source) – 20g; yeast extract – 2g; peptone – 2g; $MgSO_4.7H_2O$ – 0.5g; KH_2PO_4 – 0.46g and K_2HPO_4 – 1g in distilled water (Cho et al., 2002). Influence of different carbon sources were studied in the media containing Yeast extract(2 gL^{-1}) and peptone (2 gL^{-1}) as nitrogen sources. Dextrose, Sucrose, Lactose, Soluble starch, Fructose, Cellulose, Maltose, Mannitol, Arabinose and Rhaffinose were evaluated as carbon sources at 2% concentration. Influence of different Nitrogen sources were studied in the media containing 2% dextrose as the carbon source. Peptone (4 gL^{-1}), yeast extract(4 gL^{-1}), Beef Extract(4 gL^{-1}), Peptone + yeast extract(2+2 gL^{-1}), Peptone + Beef extract(2+2 gL^{-1}), yeast extract + Beef extract(2+2 gL^{-1}), Ammonium Sulphate(2 gL^{-1}), Ammonium Hydrogen Phosphate(2 gL^{-1}), Ammonium Chloride(2 gL^{-1}) and Ammonium Nitrate (2 gL^{-1}) were evaluated as nitrogen sources at 0.4% concentration. Subsequently influence of pH (3, 4, 5, 6, 7, 8 and 9), temperature (10,20,25,28,30,35 and 40 ^oC) and inoculum age (4, 5, 6, 7, 8, 9 and 10 days) were studied in the culture medium containing optimized concentrations of dextrose as carbon source, yeast extract and peptone as nitrogen sources. All the experiments were performed in duplicates and repeated twice.

Extracellular red pigments:

Red pigment production was indirectly evaluated by quantification of the culture filtrate at 530 nm in a UV-Visible spectrophotometer (Schimadzu UV – 1700, pharmaspec). The units were expressed as units of absorbance ml^{-1} of the broth (Lee et al., 2001). The uninoculated Potato Dextrose Broth was used as blank

Biomass estimation:

Mycelium was separated from the culture broth by centrifugation and filtration. The separated mycelial mass was washed thrice with distilled water and dried overnight at 105 ^oC in hot air oven. The dry weight was recorded as g l^{-1}.

Antibacterial efficacy of the crude pigment extract against *Ralstonia solanacearum*:

Crude pigment extract was evaluated for antibacterial activity against plant pathogenic bacteria *R. solanacearum* isolated from diseased tomato plants (Narasimha Murthy et al., 2012). Antibacterial efficacy was tested by agar well diffusion method (Shrishaet al.,2011). The bacterial suspensions were prepared and adjusted to 10^8cfu per ml and swabbed on TSA (Tryptic Soy Agar).(Ran et al., 2005). About 100 µl of the inoculum of the test pathogen was spread on to TSA plates. A 5 mm well was made in the center of the plate using a sterile cork borer. Filter sterilized (0.45microns) ethyl extract of *C. cupreum* SS-02 pigment, dissolved in Di methyl sulfoxide(DMSO) with different concentrations at 25, 50, 75 and 100µg/ml were evaluated for their efficacy to control the pathogen. 100 µl of each concentration of the pigment was loaded into the well (Fig: 1.) and the plates were incubated at 37°C for 24 h. DMSO was used as a control. The experiment was performed in duplicates and repeated twice. After the incubation, the inhibition zone around the well was recorded and expressed in millimeter (mm).

Statistical analysis:

All the results were analyzed by means of Multivariate ANOVA using SPSS for windows (SPSS Inc.) 11.5 version. Post Hoc analysis was performed using Scheffe test with significance $p< 0.05$.

Results

The Culture was identified and deposited in the National Fungal Culture Collection of India (NFCCI), with accession Number NFCCI – 3117. BLAST search performed for the sequence of ITS analysis, showed 99% homology with other strains of *C. cupreum* available in Gen bank (Fig. 2). The sequence was deposited in NCBI Genbank with accession Number KF668034.

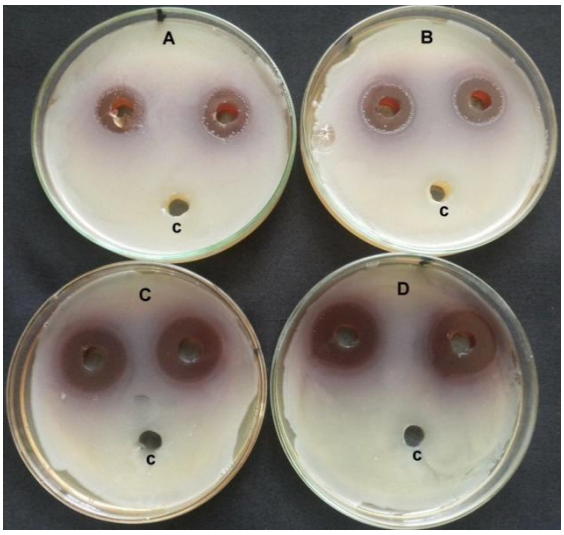

Fig: 1. Zone of Inhibition of the Pigment extract against *Ralstonia solanacearum*. A) 25µg/ml, B) 50µg/ml, C) 75 µg/ml and D) 100 µg/ml. (c : DMSO control).

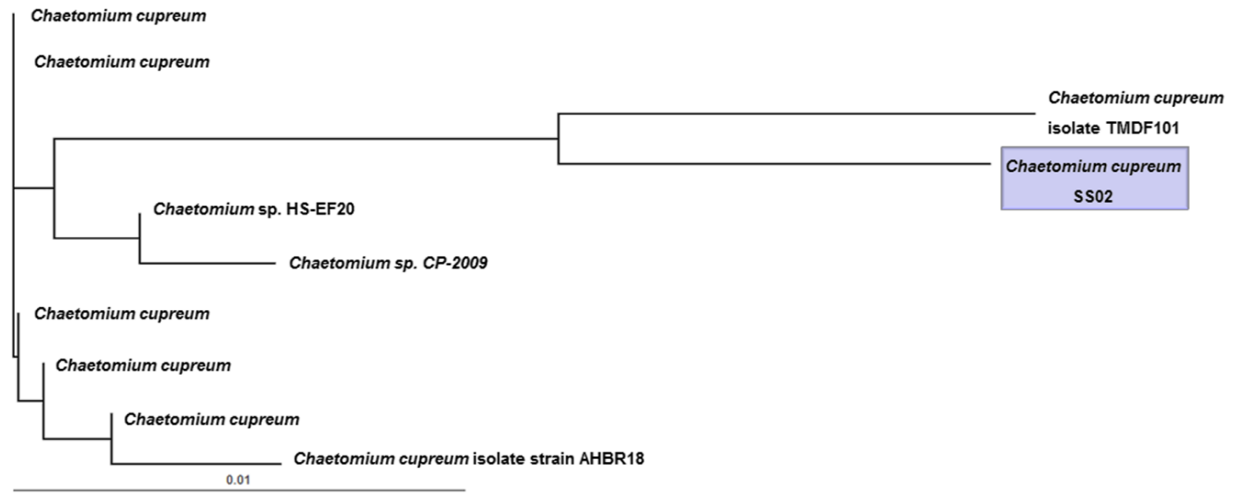

Fig: 2. Phylogenetic relationships of *C. cupreum* isolates inferred by Neighbor-Joining (NJ) bootstrap tree analysis of ITS sequences. Sequences used for this comparison were obtained under the following GenBank accession numbers: JQ676206; KF305757; GQ334427; AB506801; AB509371; AB509373; AB509369; AB509372.

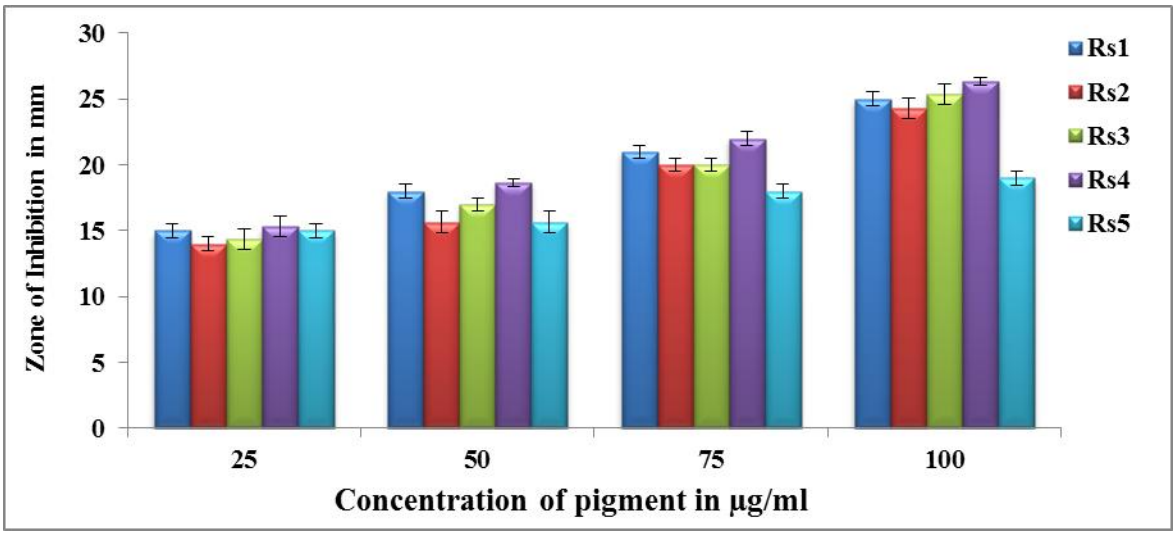

Fig: 3. Antibacterial activity of the pigment extract of *Chaetomium cupreum* against *Ralstonia solanacearum* (RS1 –RS5 strains).

Effect of Carbon source:

For the evaluation of the assimilation of sugars as carbon sources by *C. cupreum*, for its growth and pigmentation, eleven different sugars were tested. Among them, Dextrose recorded highest pigment production followed by Sucrose, Fructose, Lactose and Soluble starch (Table: 1). Pigment production was totally absent in the flasks supplemented with Cellulose, Maltose, Mannitol, Mannose, Arabinose and Rhaffinose. Highest absorbance at 530nm was recorded in the pigment extracted from sucrose and dextrose. Maximum growth was seen in cellulose and Rhaffinose exhibited the least growth.

Table: 1. Effect of Carbon sources on pigment production.

Carbon sources	Biomass in g L^{-1} ± SE	Pigment yield in mg L^{-1} ± SE	Pigment @ 530nm (UA/ ml) ± SE
Dextrose	1.57^{e} ± 0.03	563.33^{d} ± 3.48	1.64^{c} ± 0.20
Sucrose	1.56^{e} ± 0.04	556.67^{d} ± 2.96	1.68^{c} ± 0.06
Lactose	1.37^{e} ± 0.04	426.67^{c} ± 1.45	1.22^{ab} ± 0.07
Soluble starch	1.12^{d} ± 0.05	246.66^{b} ± 1.76	0.85^{b} ± 0.08
Fructose	1.49^{e} ± 0.02	473.33^{c} ± 3.76	1.46^{c} ± 0.16
Cellulose	2.06^{f} ± 0.03	0^{a} ± 0	0^{a} ± 0
Maltose	0.85^{c} ± 0.03	0^{a} ± 0	0^{a} ± 0
Mannose	0.65^{ab} ± 0.03	0^{a} ± 0	0^{a} ± 0
Mannitol	0.90^{c} ± 0.17	0^{a} ± 0	0^{a} ± 0
Arabinose	0.83^{bc} ± 0.03	0^{a} ± 0	0^{a} ± 0
Rhaffinose	0.61^{a} ± 0.02	0^{a} ± 0	0^{a} ± 0

Scheffe post hoc test: Means sharing different superscripts are significantly different ($P<0.05$).
SE = Standard Error.

Effect of Nitrogen source:

Among the various nitrogen sources, Peptone + Yeast Extract recorded highest pigment production followed by Yeast Extract, Peptone + Beef Extract, Beef Extract + Yeast Extract, Beef Extract and Peptone (Table : 2). Pigment production was totally absent in the flasks supplemented with Ammonium Sulphate, Ammonium Hydrogen Phosphate, Ammonium Chloride and Ammonium Nitrate. Peptone + yeast extract yielded the pigment with the

highest absorbance at 530nm. Maximum growth was seen in Peptone + Beef Extract and Ammonium Hydrogen Phosphate exhibited the least growth.

Table: 2. Effect of Nitrogen sources on pigment production.

Nitrogen Sources	Biomass g L^{-1} ± SE	Pigment yield mg L^{-1} ± SE	Pigment @ 530nm (UA/ ml) ± SE
Peptone	1.54^{bc} ± 0.10	116.67^{ab} ± 2.60	1.3379^{bc} ± 0.13
Beef extract	1.65^{bc} ± 0.13	146.67^{ab} ±2.96	1.2762^{b} ± 0.11
Yeast extract	1.75^{c} ± 0.10	323.33^{bc} ± 2.73	1.6275^{bc} ± 0.07
Pep+ BE	1.95^{c} ± 0.08	276.67^{bc} ± 5.24	1.5571^{bc} ± 0.09
Pep+YE	1.18^{abc} ± 0.17	470^{c} ± 7.77	1.8454^{c} ± 0.08
YE+BE	1.92^{c} ± 0.05	266.67^{bc} ± 3.53	1.4789^{bc} ± 0.14
Ammonium Sulphate	1.17^{abc} ± 0.27	0^{a} ± 0	0^{a} ± 0
Ammonium Hydrogen Phosphate	0.43^{a} ± 0.20	0^{a} ± 0	0^{a} ± 0
Ammonium Chloride	$1.14ab^{c}$ ± 0.14	0^{a} ± 0	0^{a} ± 0
Ammonium Nitrate	0.68^{ab} ± 0.16	0^{a} ± 0	0^{a} ± 0

Scheffe post hoc test: Means sharing different superscripts are significantly different (P<0.05).
SE = Standard Error.

Effect of pH:
The fungus was able to grow in a wide range of pH from 3 to 9, but produced pigments only from pH 5 to 8. Organism did not grow at pH 2 and 10, showed reduced growth at pH 4 and 9 producing very less pigment even upto 18 days of incubation. pH 6 showed the highest pigmentation absorbance and yield (Table: 3) followed by pH 7, 8 and 5. Maximum growth was seen in pH 5and pH 8 exhibited the least growth; whereas, growth was completely absent in pH 3 and 9.

Table: 3. Effect of pH on pigment production.

pH	Biomass gL^{-1} ± SE	Pigment yield mg L^{-1} ± SE	Pigment @ 530nm (UA/ ml) ± SE
3	0^{a} ± 0	0^{a} ± 0	0^{a} ± 0
4	0.34^{a} ± 0.11	0^{a} ± 0	0^{a} ± 0
5	1.77^{c} ± 0.05	263.33^{b} ± 1.05	1.1908^{c} ± 0.07
6	1.76^{c} ± 0.12	823.33^{c} ± 1.84	1.8059^{c} ± 0.09
7	$1.36b^{c}$ ± 0.14	316.67^{b} ± 1.21	$1.3897b^{c}$ ± 0.11
8	1.11^{b} ± 0.07	256.67^{b} ± 1.20	1.2243^{b} ± 0.08
9	0^{a} ± 0	0^{a} ± 0	0^{a} ± 0

Scheffe post hoc test: Means sharing different superscripts are significantly different (P<0.05).
SE = Standard Error.

Effect of Temperature:
The fungus was able to grow at all the temperatures experimented except 10 and 40^{o}C. The organism produced high yield of biomass and pigment at 35^{o}C, followed by 30, 25 and 28^{o}C (Table. 4). Incubation at 20^{o}C showed least biomass production but pigmentation was totally absent. Maximum growth was seen at 35^{o}C and 20^{o}C exhibited the least growth, whereas, growth was completely absent in 10 and 40 ^{o}C.

Table: 4. Effect of temperature on pigment production.

Temperature	Biomass g L^{-1} ± SE	Pigment yield mg L^{-1} ± SE	Pigment @ 530nm (UA/ ml) ± SE
10	0^{a} ± 0	0^{a} ± 0	0^{a} ± 0
20	0.18^{a} ± 0.06	0^{a} ± 0	0^{a} ± 0
25	0.80^{ab} ± 0.11	336.67^{b} ± 2.96	1.0377^{b} ± 0.48
28	0.55^{ab} ± 0.20	430^{b} ± 3.21	1.2467^{bc} ± 0.82
30	1.88^{ab} ± 0.03	423.33^{b} ± 2.02	1.2162^{b} ± 0.87
35	1.90^{b} ± 0.13	696.67^{c} ± 2.67	1.6831^{c} ± 0.15
40	0^{a} ± 0	0^{a} ± 0	0^{a} ± 0

Scheffe post hoc test: Means sharing different superscripts are significantly different (P<0.05).
SE = Standard Error.

Effect of Inoculum age:
The age of the inoculum showed varied pigment production. $3^{rd,}$ $4^{th,}$ 9^{th} and 10^{th} day old culture produced less pigment after prolonged incubation. 6^{th} day old culture produced highest biomass and pigment and 8^{th} day old culture produced the least pigmentation. 10^{th} day old culture exhibited the least growth (Table: 5).

Table: 5. Effect of inoculum age on pigment production.

Inoculum age In days	Biomass g L^{-1} ± SE	Pigment yield mg L^{-1} ± SE	Pigment @ 530nm (UA/ ml) ± SE
3	1.25^{ab} ± 0.16	476.67^{b} ± 1.42	1.4809^{abc} ± 0.07
4	1.64^{ab} ± 0.10	613.33^{c} ± 1.71	1.7958^{c} ± 0.08
5	1.49^{ab} ± 0.07	716.67^{cd} ± 1.79	1.6732^{bc} ± 0.08
6	1.82^{b} ± 0.07	883.33^{e} ± 1.92	1.8741^{c} ± 0.09
7	1.67^{ab} ± 0.07	783.33^{de} ± 1.84	1.6836^{c} ± 0.09
8	1.24^{a} ± 0.08	220^{a} ± 1.09	1.1507^{ab} ± 0.10
9	1.33^{ab} ± 0.07	280^{a} ± 1.30	1.4819^{abc} ± 0.09
10	1.24^{a} ±0.09	266.67^{a} ± 1.24	1.146^{a} ± 0.07

Scheffe post hoc test: Means sharing different superscripts are significantly different (P<0.05).
SE = Standard Error.

Antibacterial efficacy of the crude pigment extract against *Ralstonia solanacearum*:
The antibacterial activity of the crude pigment extract was tested against five pathogenic strains of *R. solanacearum.* The pigment inhibited all the five pathogenic strains and the highest antibacterial activity was recorded against RS4 strain and the least activity was recorded against RS2 strain. The antibacterial activity was measured as zone of inhibition expressed in millimeter (mm). The highest activity of 15.33, 18.66, 22 and 26.33mm was recorded against RS4 strain at 25, 50, 75 and 100 µg/ ml concentrations respectively (Fig. 3).

Discussion

Optimum conditions should be provided to get the best yield of the pigment. Among the eleven carbon sources tested dextrose induced highest pigment production as compared to other sugars. A stimulatory effect of nitrogen source on pigment formation has been reported (Hamdi et al., 1997) and our study on effect of nitrogen agrees with it. Same kind of observations have been reported by Chen et al., (1993), Fang et al., (1993) and Kang et al., (1996).It has also been reported that various types of peptone support greater pigment production in many kinds of pigment producing fungi (Cho et al., 2002). The pH conditions of a medium largely influences pigment yield. The findings of our study agree with the results of Sardaryan et al.,(2004). The optimal temperature for high pigment yield was

found to be 35^{o}C. Accounting to that higher fungi usually require long periods for growth in submerged culture, which exposes them to contamination risk, this optimal temperature can be regarded as a favourable physiological property of *C. cupreum.* Amongst numerous physiological properties of fungi, the inoculum age is known to play an important role in fungal development (Glazebrook et al., 1992; Bae et al., 2000). The optimization of the above culture conditions improved the pigment yield by five fold in submerged conditions. Screening of the pigment for its antibacterial efficacy against *R. solanacearum,* gave promising results, paving way for probable application as a biocontrol agent to control the tomato wilt caused by the pathogen. Our results are similar to the results of bioantagonistic agents screened by NarasimhaMurthy et al., (2012). The high yield of pigment produced by *C. cupreum* SS-02 demonstrates the possibility of commercial production of pigment by this strain. Considering its relatively high production yield, further characterization of the pigment and field trials are required to establish the pigment extract as a commercial biocontrol agent. Our study is a preliminary investigation in this path.

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