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INHIBITION OF VIOLACEIN SYNTHESIS IN *CHROMOBACTERIUM VIOLACEUM* DSTS-1 MUTANT

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ABSTRACT

Chromobacterium violaceum DSTS -1 (C-) mutant is a violacein negative and phenol degrading strain. LuxI/LuxR specific gene amplification conducted in *Chromobacterium violaceum* C- proved the existence of LuxI/LuxR homologue. The ether extract of the supernatant of *Chromobacterium violaceum* C- grown pigment production medium, gave a single spot of Rf value 0.38 in TLC. The ether extract of this scrapped spot on HPLC gave a single peak at 38.63m. FT/IR analysis followed by GCMS analysis proved that the compound is 1,2-benzene dicarboxylic acid. This might be acting as an inhibitor to anthranilate synthase, the broad substrate specific and multiple reaction catalyzing enzyme involved in the synthesis of tryptophan, which is the only precursor for violacein synthesis.

Keywords- Quorum Sensing, 1, 2-benzene dicarboxylic acid, anthranilate synthase

[I] INTRODUCTION

Bacterial cells can communicate with each other through Quorum sensing. 'Quorum Sensing' (QS) can be described as a process through which the accumulation of signalling molecules enable a single cell to sense the communication from other cells. QS helps bacteria to co-ordinate their behaviours. Many species of bacteria use quorum sensing to coordinate their gene expression based on the density of their population. Quorum sensing occurs within a single bacterial species as well as between diverse species, and regulates a lot of different processes, essentially serving as a simple communication network [10].

Bacteria using quorum sensing constantly produce and secrete certain signalling molecules known as autoinducers or pheromones. They possess specific receptors which can detect these signaling molecules. Upon binding of inducer to this receptor, it activates transcription of certain genes, including those for inducer synthesis. The *lux*-type quorum-sensing system is the pre eminent mechanism for species-specific communication in gram-negative bacteria.

Chromobacterium violaceum is a facultative anaerobic, oxidase-positive, glucose-fermenting, non-lactose-fermenting, Gram-negative bacillus. *C. violaceum* is also known for being a reporter strain in quorum sensing. It produces a possible antioxidant called violacein a purple pigment that gives *Chromobacterium violaceum* its color. [1] Violacein has been studied and proven to defend protect the membrane of microorganism,from oxidation or peroxidation.

This bacterium can also produce acid (ferment) from glucose, trehalose, N-acetylglucosamine and gluconate, but not L-arabinose, D-galactose or D-maltose. In addition, it can produce cyanide in the form of ammonium cyanide. Lastly, casein is hydrolyzed and arginine decarboxylated in the presence of this bacterium. [5, 6] *C. violaceum* is abundantly present in soil and water in the tropical and subtropical regions. They usually reside in fresh bodies of water, but not in the normal human flora. Slightly curved rods, *C. violaceum* can range from medium to long lengths, and have rounded ends. Colonies are optimally produced during incubation at 22°C while cultured in 5% sheep blood agar under anaerobic conditions. Other agars such as Mac Conkey agar, and Chocolate agar can also be used to cultivate this organism. Colonies appear black or very dark purple and give a smell of ammonium cyanide [4].

The *C. violaceum* quorum-sensing system consists of the luxI/luxR homologues CviI/CviR [7, 8].The CviI/CviR circuit controls virulence, as evidenced by the fact that antagonist moleules that bind in place of the natural AHL controls quorum sensing and these quorum-sensing inhibitors could be valuable in battling virulent bacteria [2].

The product of the shikimate pathway, chorismate, is a branchpoint for five biosynthetic pathways leading to the formation of the major aromatic metabolites, including the aromatic amino acids phenylalanine, tyrosine and tryptophan. Anthranilate synthase catalyses the first committed step in the biosynthesis of tryptophan, in which chorismate is first aminated and then the enol-pyruvyl side chain cleaved to form the aromatic product anthranilate . Anthranilate synthase is a multifunctional enzyme composed of a small TrpG and large TrpE subunit encoded by the *trpG* and *trpE* genes,respectively [3].

As anthranilate synthase is an essential enzyme involved in tryptophan synthesis,the substrate for violecein synthesis,any inhibition of this enzyme negatively influences violacein synthesis. The first series of inhibitors were the 4-methoxybenzoate analogues, a simple replacement for hydroxyl that introduces possible steric interactions. The second series were the 4-aminobenzoate analogues. In this series, two different C- 3 substituents were used; one retaining the ether linkage of chorismate and the other with a secondary amine linkage [11].

The strain *Chromobacterium violaceum DSTS -1* was available in the culture collection centre of School of Biosciences, Mahatma Gandhi University [12]. But during the storage the strain had lost the ability to synthesize violacein .We initiated the present project to explore the difference in quorum sensing regulation and violacein synthesis between the wild and mutant *Chromobacterium violecein* and also to evaluate the reason behind the inhibition of violacein synthesis in the *Chromobacterium violecein* mutant.

[II] MATERIALS AND METHODS

2.1 Microorganism and the source

Microorganisms used in the study were two *Chromobacterium violaceum* strains (C+ and C-), (i) *Chromobacterium violaceum DSTS-1 mutant(C-)* was violacein minus and was colourless. The organism was available in the culture collection centre of School of Biosciences, Mahatma Gandhi University. The culture was maintained on nutrient agar medium (ii) *Chromobacterium violaceum* (C+) (wild strain) was violacein producing violet coloured culture collected from IMTECH (Chandigarh).

2.2 Medium

The medium used in the present study for culture maintenance was modified nutrient agar medium. The composition of media was as follows: peptone 0.1g, NaCl0.1g, beef extracts 0.05g, yeast extract 0.0001g, agar 2gm, Distilled water100ml

2.3 Isolation of Bacterial Genomic DNA

Bacteria from a saturated liquid culture were lysed and the proteins were removed by digestion with Proteinase K. Cell wall debris, polysaccharides, and remaining proteins were removed by selective precipitation with CTAB, and high-molecular-weight DNA was recovered

from the resulting supernatant by isopropanol precipitation [9].

2.4 Detection of LuxI and Lux R homologus–Primer Selection

PCR detection of Lux I and LuxR homologue genes was carried out using the following primer

Primer	Sequence
Lux I- F	5' GAATTCCGCTGGGAATACAATTAC 3'
Lux I- R	5'GGATCCTTTATACTCCTCCGATGGAATTGCC 3'
Lux R-F	5'CGAACGGGGTACCCATGAAAAACATAAATGCC 3'
Lux R-R	5'CGTCGCGGATCCCGTACTTAATTTTTAAAGTA TGGGCAAT 3'

2.5 PCR Condition for the Amplification of Lux I and Lux R homologue

Optimization of PCR conditions using the above primers LuxI and LuxR genes were done for *Chromobacterium violaceum* mutant strain(C-) and also for standard *Chromobacterium violaceum* strain(C+). Annealing temperatures of 45^0- 57°C were tested for optimization. The PCR temperature program began with an initial 5-min denaturation step at 95°C; 30 cycles of 94°C for 1 min, 45^o- 52°C for 1 min, and 72°C for 2 min; and a final 10-min extension step at 72°C. All reaction mixtures were held at 4°C until analyzed. All reaction mixtures also included PCR buffer, 0.2 mM each deoxynucleotide triphosphates and 1 U of Taq DNA polymerase. All experiments included controls without any added DNA. Ten microliters of each PCR mixture was run on a 1.5% agarose gel in TBE buffer stained with ethidium bromide (0.5µg/ml) and visualized under UV light.

2.6 Pigment Extraction

Pigment Production Medium, (PPM) composition peptone-2g, NaCl-0.5g, Glycerol-2g, KNO_3- 0.1g, Distilled water- 100ml with pH-7.2 was used in the present study for pigment extraction.*Chromobacterium violaceum* DSTS mutant strain(C- strain) cultures were grown in PPM broth for 5 days at 28^oC .The cultured broth was centrifuged at 10000 rpm for 15minutes and the supernatants were collected. The pH was adjusted to 2.0 with conc. HCl and it was extracted with an equal volume of ether. The ether layer was subjected to evaporation in water bath. After evaporation, the residues were resuspended in methanol. Then this methanol suspension was subjected to TLC analysis.

2.7 Thin layer Chromatography

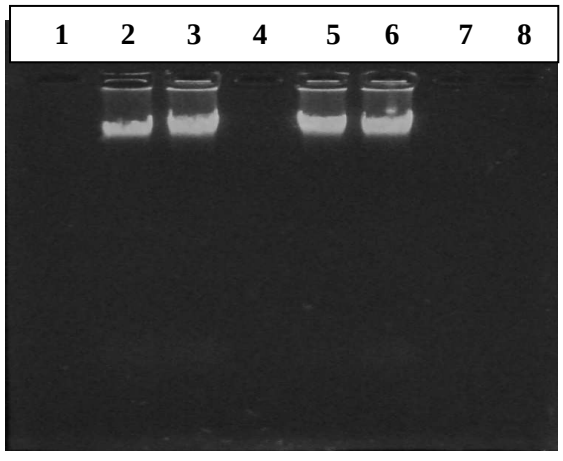
20 gm silica mixed with 40 ml distilled water. Then it was uniformly plated on a TLC plate and was kept for drying at room temperature. It was then kept in a hot air oven at 100^oC for about 1 hour .For the detection of compound, 100 µl of samples were subjected to chromatography on TLC silica gel plates. The solvents used were isopropanol/ammonia/water (8: 1: 1). The samples were allowed to run for about ¾ th of the plate. Plates were viewed under UV light and the Rf values of the spot was calculated.

2.8 FT/IR, HPLC and GCMS analysis

Spots from TLC plates were scraped and dissolved in diethyl ether. It was mixed well and was centrifuged at 10000rpm .Supernatant taken was subjected to FT/IR, HPLC and GCMS analysis. HPLC was done using SHIMADZU Analytical HPLC (School Of Environmental Sciences, Mahatma Gandhi University. FTIR analysis was done at School of Chemical Sciences, Mahatma Gandhi University, Kottayam, Kerala, India and GCMS was done at Cashew Export Promotion Council of India, Kollam.

[III] RESULTS

Fig: 1-Genomic DNA isolated and separated on 0.8% Agarose gel



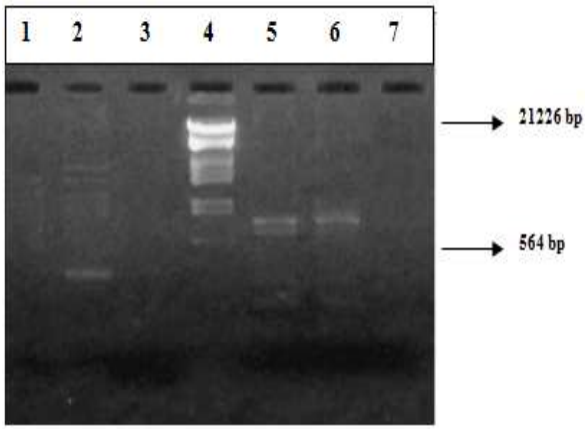
Lane 2, 3-Genomic DNA of *Chromobacterium.violaceum(C+)*

Lane 5, 6-Genomic DNA of *Chromobacterium.violaceum*,(C- mutant)

The bacterial chromosomal DNA of both *Chromobacterium violaceum* (C+) and

Chromobacterium violaceum(C-) were isolated, purified and were separated on 0.8% agarose gel (Fig 1). Lane 2 and 3 represent chromosomal DNA of *C violaceum* (C+) and lane 5 and 6 represent the genome of *C violaceum*(C+).

Fig: 2 - Amplification of LuxI/LuxR homologue in *Chromobacterium violaceum* (C+) and *Chromobacterium violaceum* (C-).



Lane 1 - LuxI of *C.violaceum* (C+)
Lane 2 - LuxI of *C.violaceum mutant* (C-)
Lane 4 - DNA marker (Eco R1/Hind III Double Digest)
Lane 5 - LuxR of *C. violaceum* (C+)
Lane 6 - LuxR of *C. violaceum* mutant (C-)

Figure -2 represents the amplification of lux I/lux R homologue in *C violaceum* (C+) and (C-) Lane 1 represents lux I homologue of C+ and lane 2,lux I homologue of C-.Lane 4 represents the Eco R1/Hind III Double Digest DNA marker . Lane 5 represents homologue of lux R and Lane 6 represent the lux R homologue of C-.

Fig: 3-Thin layer chromatography of the extract of the supernatant of *C.violaceum* (C-) grown in pigment production medium.

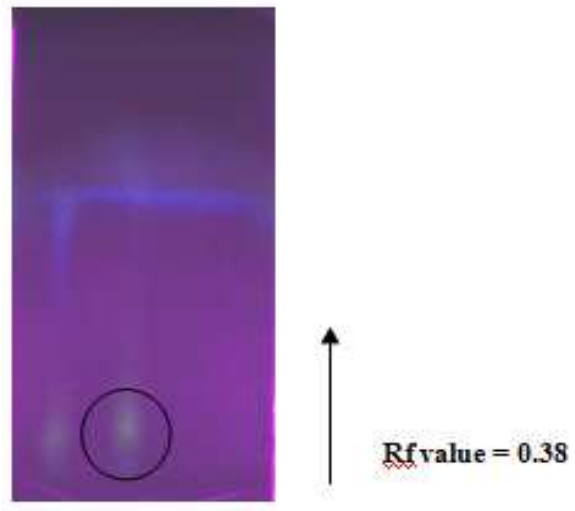


Fig: 4-FT/IR of the spot from TLC done with the ether extract of *C violaceum* grown in pigment production medium

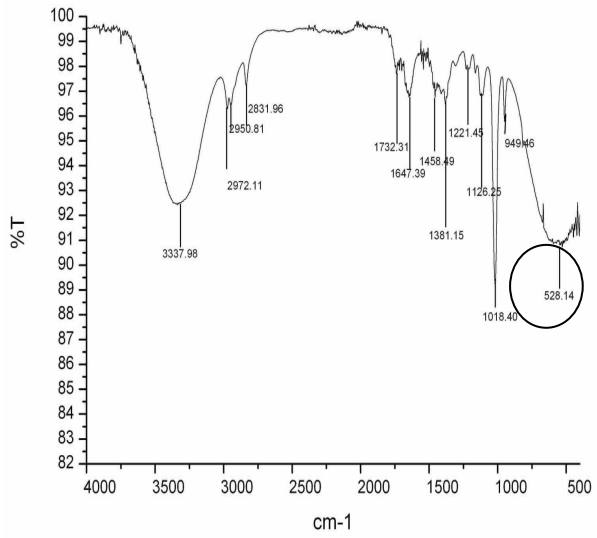


Fig.5 HPLC profile of the spot from the TLC plate done with ether extract of *C violaceum* grown pigment production medium

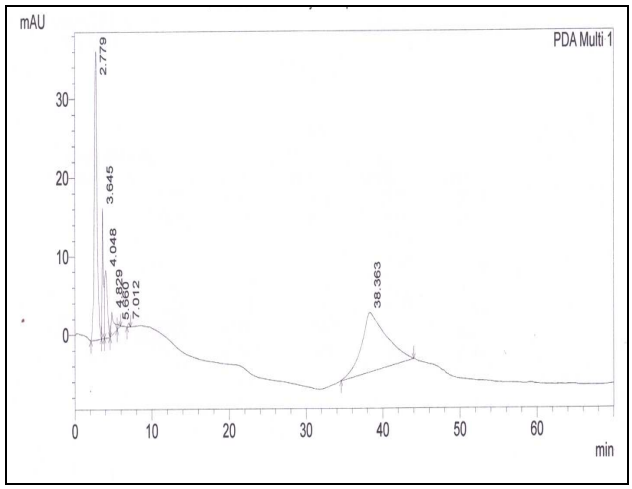
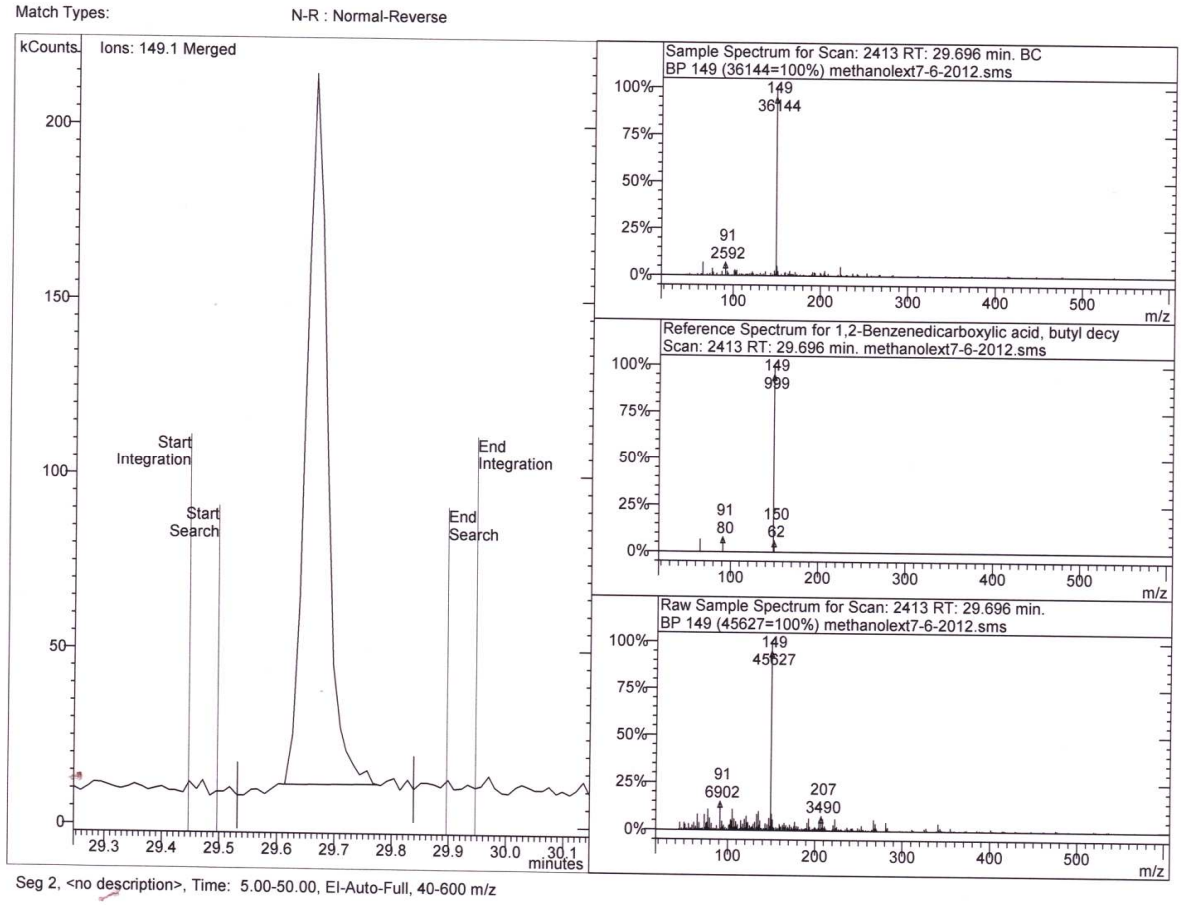


Fig. 6-MS of the predominant peak at 29.6 min in the GC of the spot from the TLC plate carrying the ether extract of the supernatant of *C violaceum* grown pigment production medium.

The spot obtained in the TLC plate was scrapped and was dissolved in ether .The ether extract was subjected to FT/IR , HPLC and GCMS .Fig 5 represents the FT/IR spectrum of the sample. Fig 5 represents the HPLC chromatogram of the sample.Fig 6 represents the mass spectrum of the most predominant peak obtained in the GC. The molecular weight of the sample is 149 and the suspected compound is 1,2-benzene dicarboxylic acid butyl decyl.



[IV] DISCUSSION

Chromobacterium violaceum is an extremely important bacterial strain with multiple industrially exploitable features. It has been utilized as the primary candidate for the detailed study of violacein pigment production, gold precipitation, cyanide production and degradation of many toxic compounds. In view of extended application in various fields of Biotechnology, Brazilian government has completed a multinational project on the genome sequencing of *Chromobacterium violaceum.*This bacterium is highly unstable and highly sensitive strain, it undergoes quick genetic mutation even on storage under refrigeration.

A *Chromobacterium violaceum* strain DSTS-1 was isolated from soil as a potent phenol degrading isolate. It could also produce the violet coloured pigment violacein extracellularly [12]. However on storage under refrigeration, the strain got mutated to violacein (-) strain. The mutated strain still exhibited the phenol degrading property. The mutant *C.violaceum* DSTS-1 got reverted through culturing in a medium containing methionine and N-acetylglucosamine. The methionine and N – acetyl glucosamine might have induced the synthesis of tryptophan. Violacein synthesis in *Chromobacterium violaceum* starts primarily with tryptophan which is formed from chorismate , the product of shikimate pathway .Violacein synthesis is very much dependent on the availability of tryptophan and if tryptophan synthesis is retained in an otherwise tryptophan mutant strain ,violacein synthesis can be reinitiated.

The present study was carried out to investigate the prime biochemical reason behind the lack of violacein synthesis in the *C.violaceum* DSTS -1 mutant. The genomic DNA of both C+ and C- mutant (violacein negative) was extracted and was separated on agarose gel electrophoresis (fig.1). Both the genomes were analysed for the presence of Lux I/LuxR homologue (figure-2).The LuxI/LuxR and its homologues based

quorum sensing is the most eminent functional mechanism in gram negative species. This is based on the production of AHL derivatives .The *C.violaceum* quorum sensing consists of lux I/luxR homologue CviL/CviR.On screening both C+ and C- strains for LuxI/LuxR analogues with the specific primers it became evident that the lux I/lux R homologue system is expressed in C- even-though it is non violacein producing (fig-2). It follows that the inhibition of violacein synthesis in C-mutant is not quorum sensing related and hence it was also not due to lack of AHL's.

The C-mutant was cultured in pigment production medium for 5 days and the culture supernatant was extracted with ether .The ether extract was seperated on TLC. A single spot was obtained after spraying with diazotized sulphanilic acid. The Rf value was calculated as 0.38. The spot was scrapped and was re-dissolved in ether. The ether extract was subjected to HPLC. A single peak obtained at 38.3 minutes states that the fraction is almost pure. The same sample was subjected to FT/IR spectroscopy. The spectrum obtained was analysed and the bands obtained are identified .There were representations regarding benzene ring, C= O,carboxylic acid ,O-H,stretching, O-H binding and –C-OH bonds.

The same sample was subjected to gas chromatography and the chromatogram showed peak with largest area as the 29.662 minutes. This peak was analysed through mass spectrum. The mass spectrum obtained showed molecular peak ion corresponding to a molecular weight of 149. Comparison with the NIST library suggested that the compound under investigation can be a dicarboxylic acid derivative of benzene (Figure 6).

Many such aromatic acid derivatives were proved earlier as possible inhibitors of violacein synthesis. Most of these inhibitors affect the tryptophan synthesis pathway from the chorismate and the prime enzyme usually affected is anthranilate synthase. Anthranilate synthase catalyses the first committed step in the biosynthesis of tryptophan, in which chorismate is first aminated and then the enol-pyruvyl side chain is cleaved to form the aromatic product anthranilate . Anthranilate synthase is a multifunctional enzyme composed of a small TrpG and large TrpE subunit encoded by the *trpG* and *trpE* genes,respectively. The first reaction catalysed by TrpE is the nucleophilic attack at C-2 of chorismate with ammonia produced by the TrpG subunit to give an intermediate, 2-amino-2-deoxyisochorismate (ADIC) The second reaction is the elimination of pyruvate from ADIC to produce anthranilate which then finally gives tryptophan which is the only precursor for violacein synthesis.

As anthranilate synthase is an essential enzyme for tryptophan synthesis, any inhibition on this negatively influence violacein synthesis. The first series of inhibitors were the 4-methoxybenzoate analogues, a simple replacement for hydroxyl that introduces possible steric interactions. The second series were the 4-aminobenzoate analogues. In this series, two different C- 3 substituents were used; one retaining the ether linkage of chorismate and the otherwith a secondary amine linkage .The final series of compounds proposed as targets were the 4-hydroxybenzoate analogues. These analogues most closely mimic chorismate, but with the secondary hydroxyl at C-4 replaced by a more acidic phenolic hydroxyl. Again, two types of side chains were used at C-3. One contained an ether linkage as found in chorismate. The other series of analogues incorporated an additional methylene unit before the ether linkage on the C-3 side chain. Inspection of the active site of anthranilate synthase indicated that there was unoccupied space where the enol-pyruvyl side chain of chorismate binds, which could be explored by extending the side chain. The second step of the reaction catalysed by anthranilate synthase involves elimination of the enol-pyruvyl side chain. It was envisaged that extension of the side chain may mimic this departure. The dicarboxylic acid derivative of benzene produced during the growth of *C.violaceum* (C-) in pigment production medium might have

inhibited anthranilate synthase and could have stopped tryptophan synthesis .This could be the reason for the lack of tryptophan synthesis and hence also for the lack of violacein synthesis.

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