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Isolation and Biochemical Characterization of Anti Neoplastic bacteria obtained from Domestic Liquid Waste: A potential Source of *Janthinobacterium lividum*

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Abstract

Exploration of the microbial community from the domestic sewage was the principal area of research. The domestic liquid sewage was utilized as a test to contemplate the different variety of the microorganisms present in it. Morphology, growth and pigmentation attributes of the various microorganisms were studied with the assistance of biochemical tests. A screen for antibiosis identified an atypical pale blue-purple producing bacteria, designated as JS-1 and related to Janthinobacterium lividum. Different types of culture media (NA, TYEG, TSA, and LB) were used to isolate and grow the bacteria of interest, JS-1 and it is a gram negative bacilli form capnophillic bacteria. According to the findings, strainJS-1 should indeed be classified as a new strain of Janthinobacterium.

Keywords: Domestic sewage, *J. lividum*, antibiosis, TYEG, Capnophilic, strain.

Introduction

Wastewater is produced by domestic sewage, agricultural operations, and industrial waste, and 90.62 cent of the overall wastewater generated eventually end up in coastal seas untreated. The typical residential wastewater contains organic materials, nitrogenous chemicals, suspended particles, dissolved oxygen, and bacterial colonies[1]. Microorganisms are ubiquitous in nature and perform a variety of critical tasks. Many bacteria have evolved to thrive in certain environmental niches and have been incredibly valuable in finding solutions to several problems encountered by mankind in maintaining the quality of the environment, human and animal health[2,3]. Patna is located between 24°97'-25°27'N latitude and 84°44'-86°57'E longitude[5]. Domestic waste refers to the waste material discharged from houses comprising food wastes like (vegetables, meat and dairy leftovers) classified as wet garbage, including some dry garbage which usually alters the soil and water quality but provides a nutrient-rich niche for diversified bacterial growth[6]. Types of domestic waste, Neighbor population, their activities and location affect the microbial property of water a lot[7]. Bacterial strains belonging to the class betaproteobacteria and the genus Janthinobacterium has been most frequently isolated from soil and water ecological systems[4]. The family Oxalobacteraceae includes *Janthinobacterium lividum*. The newly found *J. violaceinigrum*, *J. aquaticum*, and *J. rivuli* are among the 13 genera in this family, so as *are J. lividum*, *J. svalbardensis*, and *J. agaricidamnosum*[8]. *Lividum is a Gram-negative, Capnophilic, purple-violet bacilliform bacterium*[9]. The species was called after the Latin word janthinus, which means "violet", due to its distinctive violet color. Pigmentation results from the compound violacein formed during metabolism by the organism's biochemical activity. This violacein production reduces the microorganism's environmental constraints and strengthens its defense against external dangers[10]. Furthermore, it is understood that violacein has antibacterial, antineoplastic, antiviral, and antifungal properties[11]. Wilkinson et al. recently established the therapeutic potential of violacein and its derivatives against *P. falciparum*, indicating anti-parasitic action[12]. The current study focuses on the microbial status of water samples and the isolation of anti-oncogenic bacteria that produce violacei Negative Resultlike pigment from Meta-genomic analyzed samples of domestic liquid waste, as shown in Table-8 and collected from a densely populated area of eastern India, PMCH (25°37'19.12"N, 85°10'07.85"E), Patna, Bihar[13]. The aim of this analysis was to describe the taxonomy of a single *Janthinobacterium* isolate depending on the physical and biochemical properties.

Materials and methods

All analytical work was done at the Department of Biotechnology (Lab of Environment and Biotechnology-LOEB) laboratory, Patna Science College, Patna.

Sample collection: Water sample was collected from the populated locality of PMCH (need to be elaborated/referenced). The sample was collected in the morning time in pre-sterilized glass bottles with a capacity of 500ml and was further used for isolation of bacteria. The samples were kept in sterilized plastic bottles at 4°C till further use.

Sample processing: The Sample was processed immediately upon arrival using aseptic techniques, it was Serial Diluted upto 10^8 times by pipetting out 1ml of processed sample to make working solutions of (10^{-3},10^{-4},10^{-5},10^{-6}) dilutions respectively as per standard protocol. The Original sample (OS) was greyish with a pungent smell having a pH of 7.9. The OS filtered using Whatman filter paper of standard size was used to obtain clear working solution (Control) which was subjected to determine dissolved oxygen (DO) as per the standard protocol and as a result, indicated in Table-7, the sample was found polluted[14]. All procedures are carried as per International Standard Protocol.

To support the growth of bacteria, present in the sample taken and to isolate the pure strain of the bacteria of interest, four different types of Culture Media were used.

Nutrient Agar Media (NA Media):0.5g of Peptone, 0.3g of Beef extract and 0.15g of Agar were taken and dissolved in 100 ml of distilled water followed by boiling it on a hot plate to form a uniform solution which was subsequently autoclaved.

Tryptic Soy Agar Media (TSA Media): 10g of Tryptic Soy Agar powder was suspended in 100ml of distilled water followed by boiling the mixture to dissolve the medium completely which was subsequently autoclaved and the uniform solution obtained was then allowed to cool. Lastly it was mixed well and poured into sterile Petri plates.

Lysogeny Broth / Luria-Bertani Media (LB Media):1gram Tryptone, 0.5gram Yeast Extract, 1gram NaCl and 1.5 gram Agar was diluted in 100ml of distilled water followed by boiling it on a hot plate to form a uniform solution which was subsequently autoclaved and the uniform solution obtained was then allowed to cool.

Tryptone Yeast Extract Glucose Media (TYEG Media): 1gram Tryptone, 0.5gram Yeast Extract, 1gram NaCl was dissolved in 100 ml of distilled water followed by boiling it on a hot plate to form a uniform solution which was subsequently autoclaved and solution obtained was then allowed to cool.

The media was prepared with accurate measurements and uniformity in order to avoid overgrowth of bacterial colony that makes it difficult to isolate the specific bacteria of interest, designated as JS-1.

Isolation of Bacteria: 1ml of the aliquot from each dilution was distributed on the nutrient agar plates. Following that, diluted water samples were streaked onto various agar media (i.e., Luria–Bertani (LB), TSA (Tryptic Soy Agar), and TYEG (tryptone yeast extract glucose media)). Then plates were under incubation at 18^{o}C for up to 3 weeks in capnophilic conditions-(5%CO_2, 10%O_2, 85%N_2, Salt:1-2%, pH-4 to 10). After three weeks of incubation at 18^{o}C and pH-6 among others, light bluish-violet colonies appeared on 10^{-6} diluted TYEG agar plates. Cells with this serotype were streaked repeatedly on LB and NA media plates before being sub-cultured in static LB-Broth until pure cultures of standardised colonies were recovered. One out of these was selected for further study and given the name JS-1 as shown in Figures-6,7. Other morphologically different colonies were purified by sub-culturing through streaking and serial dilution. These were subjected to several biochemical and morphological tests for further identification. For further use, each pure isolates were maintained as stock culture in slants at 4^{o}C.

Morphological test: The structure and scale of JS-1 cultures in the log phase of growth were examined under a light microscope at 100x magnification. Silver impregnation procedure was utilised to search for the presence of flagella[15]. The best temperature and pH for the cultures' growth were determined using conventional technologies. The LB plates were supplemented with NaCl concentrations ranging from 1% to 3% to measure salt resistance. A streak plate of fresh culture was observed after overnight incubation at 18^{o}C, and colony morphology was determined for morphological characterization. The shape, elevation, texture, color, and optical properties were used to assess morphological characteristics of bacterial colony. The Gram Staining Technique was used to classify the cultural features, and the cellular structure was analyzed under a light microscope at 100x magnification[16].

Biochemical Test: The cultures were grown at 18^{o}C in the required media for all experiments. Catalase, oxidase, phosphatase, gelatinase, beta-galactosidase, and urease activities were performed according to standard protocols[17]. As previously mentioned, indole production, citrate consumption, nitrate reduction to nitrite, casein and esculin hydrolysis were all calculated[18,19]. The standard methods for methyl red screening, the Voges Proskauer test, and polyhydroxy butyrate accumulation were used[20,21]. A 0.2 percent (w/v) carbon supply containing glucose and citrate-free minimal media A was used to determine the utilisation of carbon as the primary source. The glucose oxidation and fermentation test(O/F) and the ability to create acid through carbs and related chemicals in aerobic and anaerobic conditions were screened using Hugh and Leif son's technique[22].

According to APHA (American Public Health Association, 1998), all samples were tested for MPN and a biochemical test (IMViC test)[23]. To estimate total and fecal coli type, MPN (Most Probable Number) was used (Table-6). For the detection of isolated bacteria, several biochemical tests as shown in Figure-1 was performed, including:

Indole Test: This test uses Kovac's reagent to identify bacteria converting tryptophan into indole. Positive results appear red or red-violet on the surface of a broth medium, while negative results appear yellow[24].

Simmon's Citrate Test: Citrate is a critical part of the Krebs cycle. The use of citrate by bacteria was suggested by a transition in medium color from green to deep blue[25].

Methyl Red Test: This test aims to see whether the bacteria can oxidize glucose and generate acetate or lactate. Red color indicates positive and yellow color shows negative[26].

Voges-Proskauer Test: Acetoin is a byproduct of glucose synthesis, and this examination indicates its existence. It is positive if the culture turns pink to a cherry red color, while it is negative if the culture turns yellow to a copper color[27].

Results and discussion

The current research aids in evaluating the microbial properties of domestic liquid waste from the area described in the (Table-6). All morphological features and the sample's biochemical test results and potential bacterium discoveries are described in (Tables-1,2,5) and (Figures-2,3,4,5). The sample has a high concentration of bacteria, and the findings show that a variety of bacteria heavily pollutes household liquid waste. According to the findings, *Micrococcus, Staphylococcus, and Enterobacter* bacteria are all relatively dominant. The presence of *Klebsiella, M.luteus, Bacillus spp., S. aureus, S. epidermidis, M. roseus, and Enterobacter spp.* in the water sample was confirmed by microbial analysis. Contamination with the pathogenic bacteria mentioned above may be dangerous to one's health. It is recommended that the surrounding environment be free of contamination and that all household or kitchen wares be thoroughly washed, since this can encourage the development of pathogenic microorganisms. Monitoring and analyzing the microbial content of domestic liquid waste in the laboratory on a regular basis will reveal the presence of various microorganisms. In addition, an unidentified bluish-violet JS-1 bacterial colony was isolated from eight isolates and was cultured on TYEG media before being re-cultured and harvested on LB Agar plates for studying. It's morphological and biochemical characteristics along with pigmentation production. Characterization of the isolate JS-1 revealed that the bacteria is Gram-negative, capnophillic and produce flat, irregular colonies on agar medium. While contrasting the segregate JS-1 and the attributes of type strain of *Janthinobacterium lividum* (ATCC=12473, NCTC 9796), the bacterial properties of the isolate JS-1 is conforming to the recognizable proof keys[28]. Consequently, the isolate is probably positioned under *Janthinobacterium* spp. J. *lividum* species are gram-negative bacteria that ordinarily produce violet pigment on strong media and are considered solid and water organisms[29,30]. Strains of J. *lividum* isolated so far from water and soil of rivers, lakes, springs and decomposing wastes constitute an only small percentage (5 percent of total capnophillic heterotrophic bacterial population) hence the present investigation was carried out with a sample from domestic sewage water[31]. *J. lividum* is notable for producing a diverse array of bioactive compounds. These biologically active compounds are beneficial in a variety of biotechnological applications. Due to a paucity of whole genome sequences, comparable genomics of J. lividum is limited in its ability to explore overall genomic organisation and evolution[32]. Violacein extracts are now commercially accessible (Cas. number, 548-54-9), but Using isolated wild-type strains to mass-produce violacein on a commercial scale has various drawbacks. As a result, a novel method of heterologous violacein synthesis via metabolic and enzyme engineering has been predicted. However, its host production system requires holistic optimization to obtain a sustainable and balanced model. Subsequently, the low solubility of violacein limits its further applications. However, recently, Berti et al. and Choi et al. revealed that surface-active ionic liquid complexes may dissolve violacein in micellar aqueous environments and membrane vesicles can increase the solubility and transportation to target cells thus increases the bactericidal and cytotoxic activity upto many folds[33,34]. Several regulatory proteins are involved in gene regulation and auto-induction for violacein synthesis, however, further study is required to evaluate the consequences of these regulatory mechanisms on violacein synthesis and to expand the use of heterologous production systems to achieve better productivity and potency. Furthermore, for future violacein research, the usage of violacein as a biopolymer is expected to expand. Nevertheless, the targeted treatment of skin and intestinal inflammatory disorders and infestations, as well as the use of violacei Negative Result based biosensors, are also of considerable interest[35].

Table-1: Morphological Characteristics of Bacterial Isolates.

Isolates	Media	Dilution.	Plating	Colour	Texture	Margin	Elevation	Gram stain	Colony Shape
A	TSA	10^{-6}	Streak	Pink	smooth	Entire	Raised	Positive	Regular
B	TSA	10^{-6}	Streak	Grey	smooth	Entire	Raised	Positive	Regular
C	TSA	10^{-6}	Streak	Yellow	creamy	Entire	Flat	Positive	Circular
D	TSA	10^{-6}	Streak	Glittery pink	Dry	Entire	Flat	Negative	Regular

E	NA	10^{-6}	Streak	Pale yellow	smooth	Entire	Flat	Positive	Irregular
F	LB	10^{-6}	Streak	White	Slimy	Entire	Raised	Negative	Irregular
G	NA	Control	Spread	Yellow White	Moist	Entire	Raised	Positive	Circular
JS-1	TYEG	10^{-6}	Streak	Blue-Violet	Slimy	Entire	Flat	Negative	Irregular

*TSA: Tryptic Soya Agar, NA: Nutrient Agar Media, LB: Luria-Bertani Media, TYEG: Tryptone Yeast Extract Glucose Media.

Table-2: Biochemical Characteristics of Bacterial Isolates.

Isolates	Citrate Test	Indole Test	Methyl Red Test	Voges-Proskauer Test	Bacteria	Shape
A	Negative Type Strain	Negative Type Strain	Negative Type Strain	Negative Type Strain	*M. roseus*	Cocci
B	Negative Type Strain	Negative Type Strain	Negative Type Strain	Positive Type Strain	*S. epidermidis*	Cocci
C	Positive Type Strain	Negative Type Strain	Negative Type Strain	Positive Type Strain	*Bacillus*	Rod
D	Positive Type Strain	Negative Type Strain	Positive Type Strain	Negative Type Strain	*Klebsiella*	Rod
E	Negative Type Strain	Negative Type Strain	Positive Type Strain	Negative Type Strain	*S.aureus*	Cocci
F	Positive Type Strain	Negative Type Strain	Negative Type Strain	Positive Type Strain	*Enterobacter*	Rod
G	Negative Type Strain	Negative Type Strain	Negative Type Strain	Negative Type Strain	*M.luteus*	Cocci

Table-3: Standard Growth Characteristics Tests Performed for Comparative Analysis of Isolates.

Growth Characteristics	*J.lividum* (ATCC-12473)	*J.lividum* (NCTC-9796)	Isolate JS-1
Growth At 30^{o}c	Negative Result	Positive result	Negative result
Growth At 18^{o}c	Positive Result	Positive result	Positive result
pH 4	Negative Result	Positive result	Positive result
pH 6	Positive Result	Positive result	Positive result
NaCl 0.5%(w/v)	Positive Result	Positive result	Positive result
NaCl 2%(w/v)	Negative Result	Positive result	Positive result
NaCl 3%(w/v)	Negative Result	Negative result	Negative result
Sole Carbon Sources: Arabinose Citrate Fructose Galactose Glycerol Glycogen Mannose Sucrose Lactose	 Positive Result Positive Result Positive Result Positive Result Negative result Negative result Positive Result Positive Result	 Positive result Positive result Positive result Positive result Positive result Negative result Positive result Positive result Positive result	 Positive result Positive result Positive result Positive result Positive result Negative result Positive result Positive result Positive result
Acid Production From 1%: Trehalose Arabinose	Negative result Negative result	Negative result Positive result	Negative result Positive result

Galactose Glucose Sucrose Fructose	Positive result Positive result Positive result Positive result	Positive result Positive result Positive result Positive result	Positive result Positive result Positive result Positive result
Gram Stain	Negative result	Negative result	Negative result
Shape	Rod/Bacilliform	Rod/Bacilliform	Rod/Bacilliform
Motility	Positive Result	Positive Result	Positive Result
Flagella	Positive Result	Positive Result	Positive Result

Table-4: Standard Biochemical Assays Performed for Comparative Analysis of Isolates.

Biochemical Tests	*J.Lividum* (ATCC-12473)	*J.Lividum* (NCTC-9796)	Isolate JS-1
Hydrolysis of Esculin	Positive result	Positive result	Positive result
Hydrolysis of Casein	Negative result	Negative result	Negative result
Gelatinase	Positive result	Positive result	Positive result
Phosphatase	Positive result	Positive result	Positive result
Generation of H_2S	Positive result	Positive result	Positive result
Generation of PHB	Positive result	Positive result	Positive result
Reduction of Nitrate	Positive result	Positive result	Positive result
Catalase	Positive result	Positive result	Positive result
Indole	Negative result	Negative result	Negative result
Methyl Red	Negative result	Negative result	Negative result
Oxidase	Positive result	Positive result	Positive result
Voges-Proskauer	Negative result	Negative result	Negative result
Urease	Negative result	Negative result	Negative result
B-Galactosidase	Negative result	Negative result	Negative result

Table-5: Characteristics of pigment producing bacteria isolate, JS-1.

Characters Studied	Characteristics of J. *lividum* type strain.	Characteristics of isolate of JS-1
Shape of the Cell	Bacillus	Bacillus
Colony Type	Flat	Flat
Growth at 37°C	Nil	+/-
Gram's Reaction	Gram-Negative	Gram-Negative
Colour of the Pigment	Bluish Purple	Bluish Purple

Table-6: Results Represent the MPN value of Sample.

Sample	Area of Collection	MPN	MPN Index
Original Filtered Sample	PMCH, Patna	5-5-5	>1,600

Table-7: Calculation of dissolved Oxygen by Winkler method at 25^{o}C.

Dissolved O_2in Given Sample	Reading - 1	Reading -2	Reading - 3	Average
Domestic Liquid Waste	6.8 mg.ml^{-1}	5.5 mg.ml^{-1}	6 mg.ml^{-1}	6.1 mg.ml^{-1}
Normal Range	8 – 10 mg.ml^{-1}	8 – 10mg.ml^{-1}	8 – 10 mg.ml^{-1}	8 – 10 mg.ml^{-1}

Table-8: A list of bacteria found at the species level in a 16S RNA gene-based Metagenomic analysis of a residential liquid waste sample collected at PMCH (25°37'19.12" N, 85° 10' 07.85" E) Patna, Bihar, India[13].

Phylum	Class	Order	Family	Genus	Species	Absolute Count	%
Proteobacteria	*Gammaproteobacteria*	*Pseudomonadales*	*Moraxellaceae*	*Acinetobacter*	*Johnsonii*	142	0.24
	Betaproteobacteria	*Burkholderiales*	*Oxalobacteraceae*	*Janthinobacterium*	*Lividum*	138	0.23

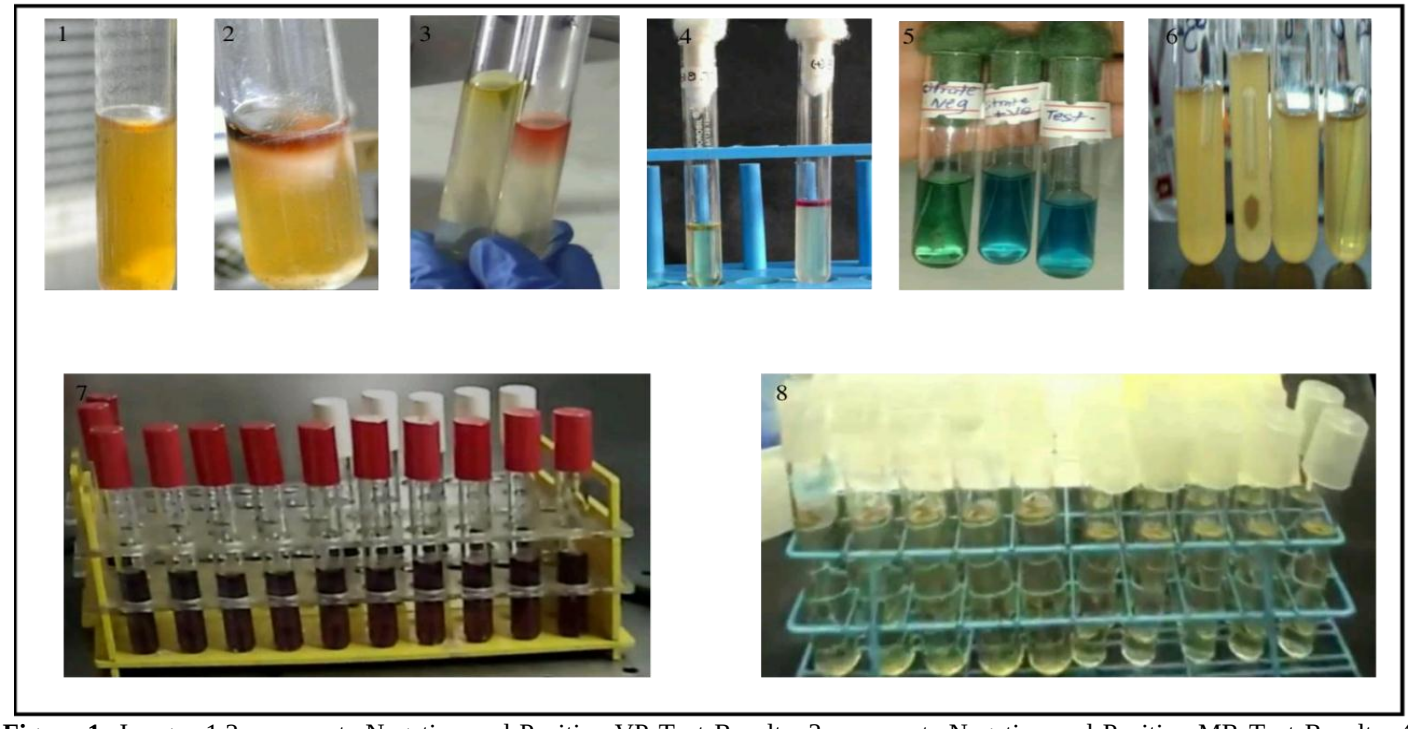

Figure-1: Images 1,2 represents Negative and Positive VP Test Results, 3 represents Negative and Positive MR Test Results, 4 represents Negative and Positive Indole Test Results, 5 represents Negative and Positive Citrate Test Results, and 6, 7, 8 represents Positive MPN Test Results.

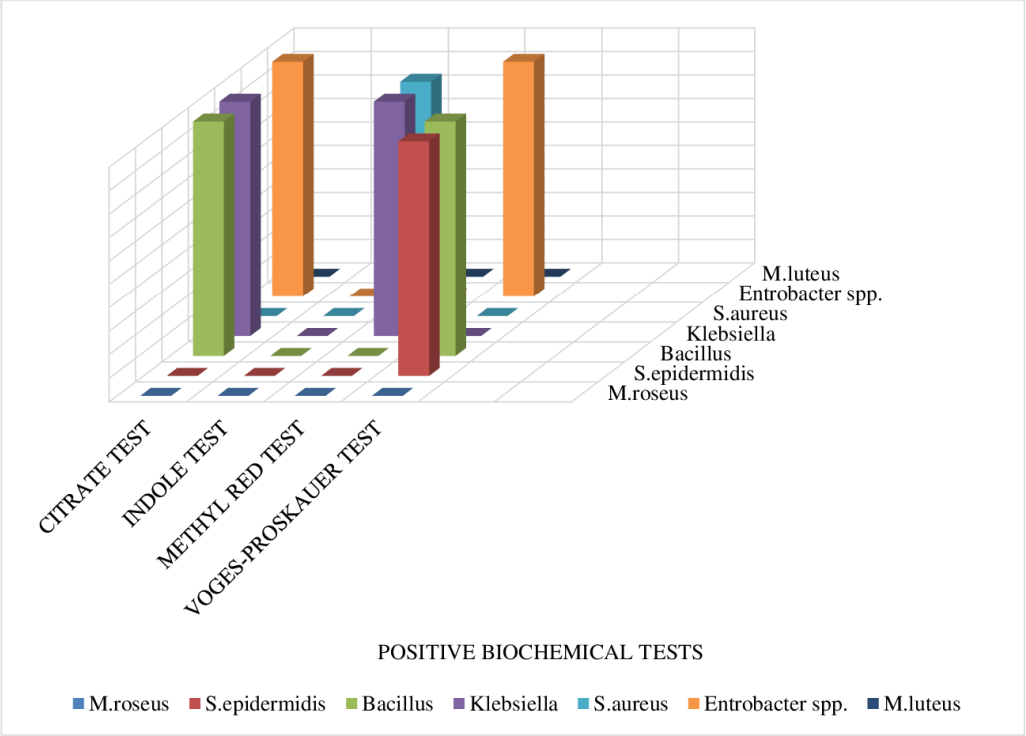

Figure-2: Bar graph indicating positive results of biochemical tests of bacterial sample as Indicated in Table-2.

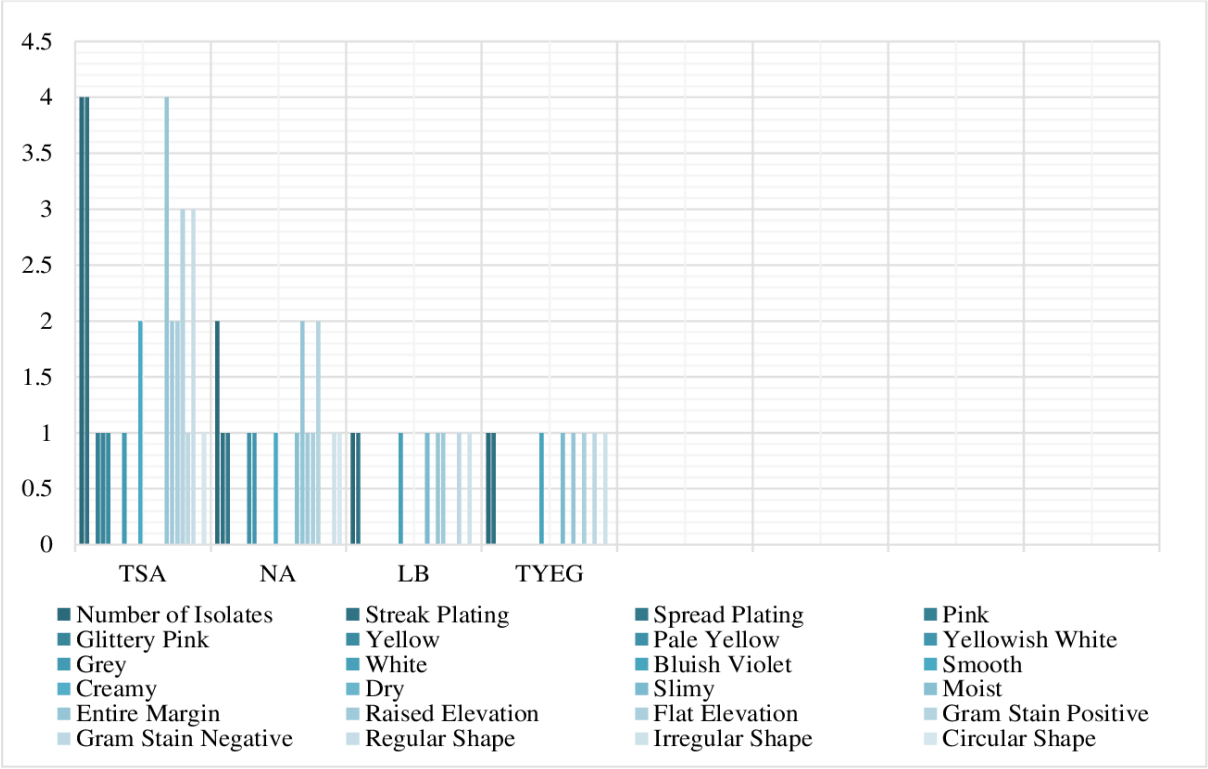

Figure-3: Bar Graph indicating Various Morphological Characteristics of isolates cultured on four different Culture Media as Indicated in Table-1.

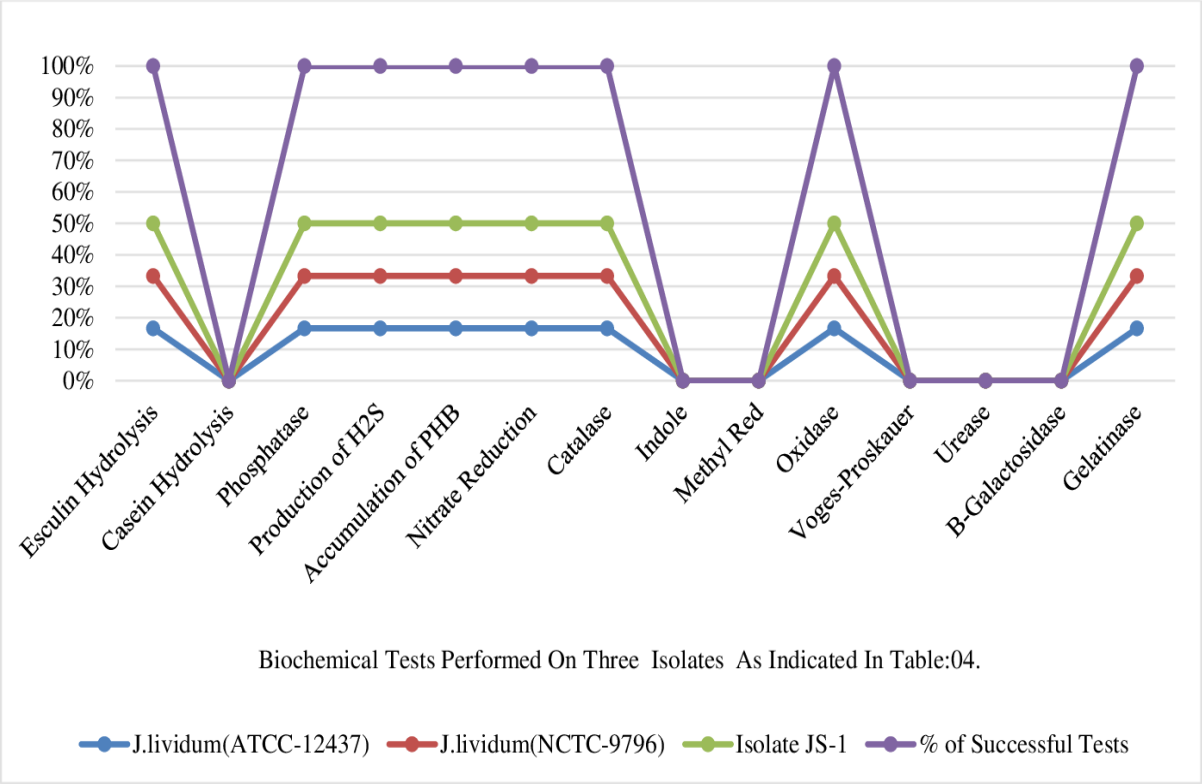

Figure-4: Line graph indicating Biochemical tests Performed with Respective Percentages of Positive Results.

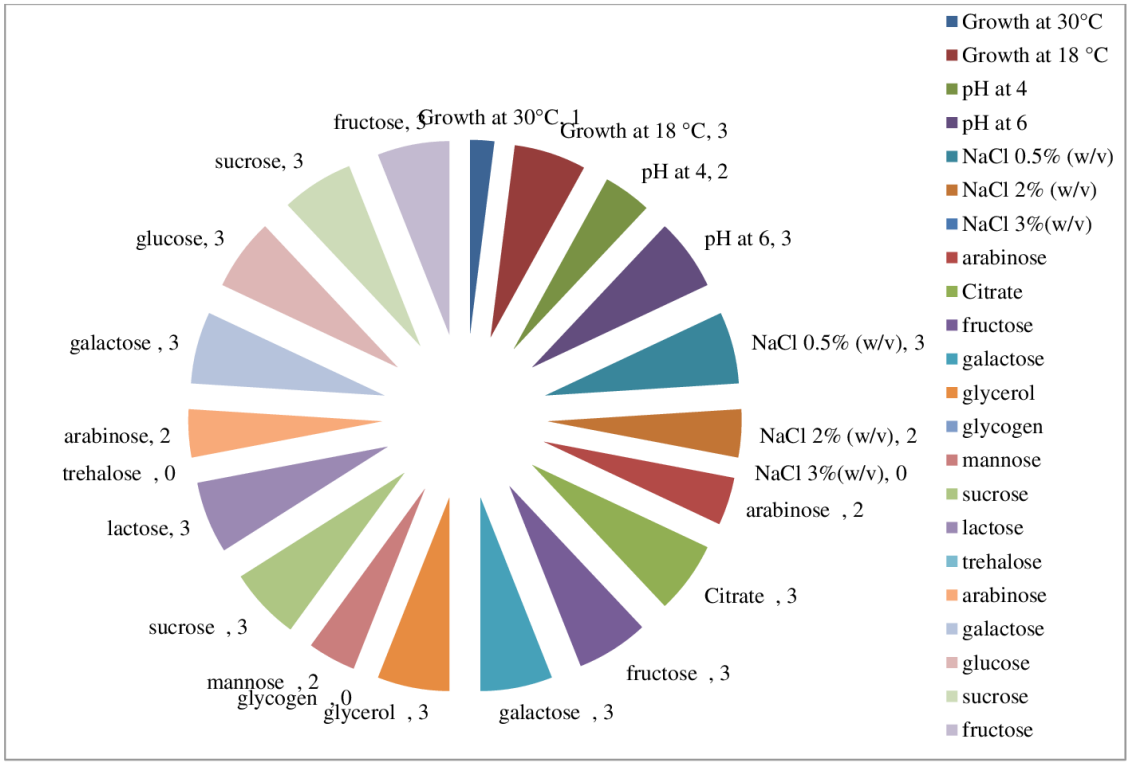

Figure-5: Pie Chart Indicating Number of Isolates Showing Positive Results for tests performed to Study "Growth Characteristics" Under Given Physiological Stress out of total three Isolates as Indicated in Table-3.

Figure-6: Mixed Culture of Bacteria on TSA.

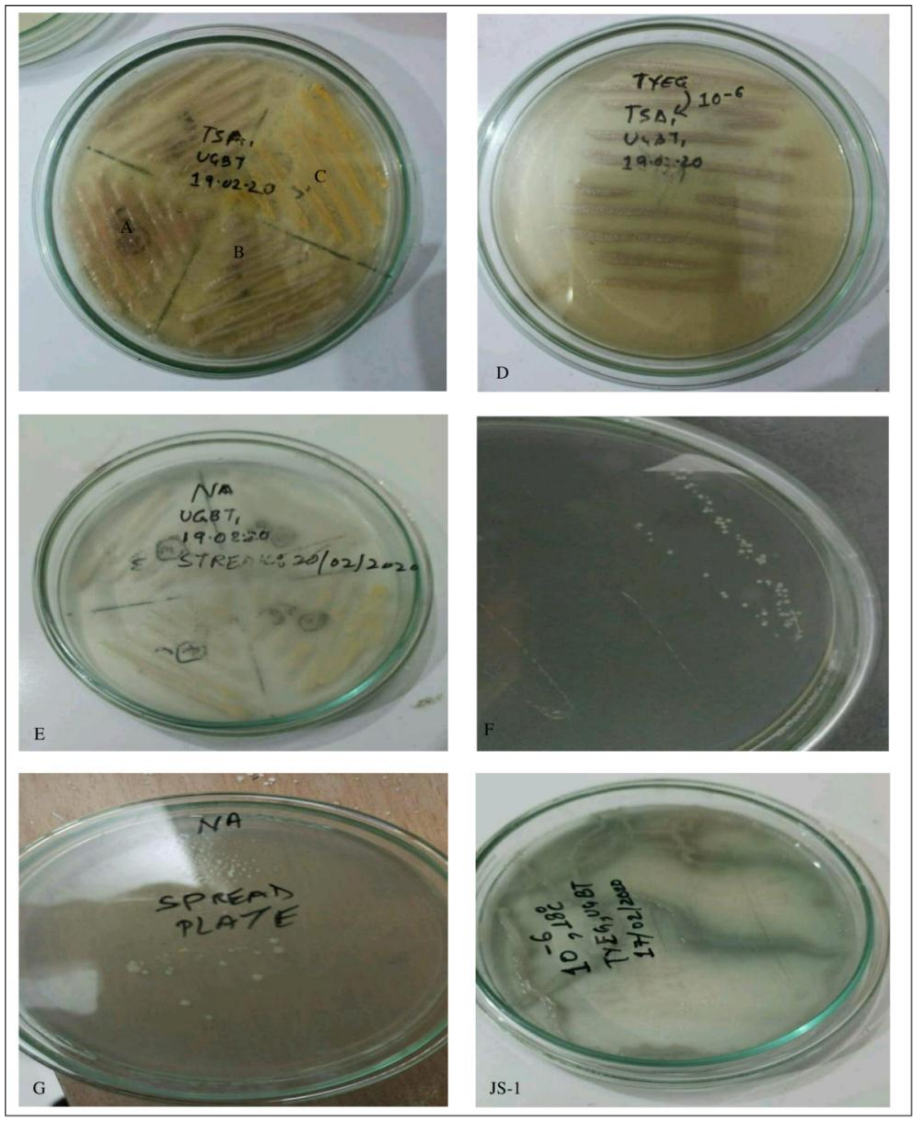

Figure-7: Bacterial Growth Shown in Petri Plates.

Conclusion

Bluish purple pigment producing bacterium secluded from decomposed sewage liquid was biochemically identified as *Janthinobacterium* sp. JS-1. The bacteria produce color ranges from blue to purple. The ease with which the bacterium can be cultured has added benefits in terms of developing an economically feasible and viable technology for the production of colorful metabolite violacein for treatment purposes. However, more study into the optimization of pigment synthesis and its biosynthetic process is required.

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