

Evaluation of Prodigiosin pigment for antimicrobial and insecticidal activities on selected bacterial pathogens & household pests

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Abstract— Bacterial pigments are gaining commercial importance in various industries like food, textile, cosmetics, pharmaceutical and other industries. Bacterial pigments are advantageous over others as they are cost effective and eco friendly. Prodigiosin has been reported to have many applications such as antibacterial, antifungal, tumor suppressor, antimalarial, antiprotozoal, immunosuppressive etc. due to its natural red pigmentation. Applications of prodigiosin prompts one to develop a cost-effective bioprocess for its production. Multi-facilitated potency of prodigiosin pigment produced by *Serratia* could be explored not only for antibacterial and antifungal but also as effective biocontrol agent in agriculture. Present study therefore aimed to obtain a potential isolate of *Serratia* for prodigiosin production. Evaluation of the pigment was carried out with four bacterial strains, two fungal plant pathogens and four insect pests.

Peanut broth when inoculated with the isolate could yield maximum pigment production at 30⁰C for 72hours. The extracted pigment purity was confirmed by the spectrophotometric method and also by TLC. Prodigiosin was observed to be effective against *E.coli* and *Staphylococcus* with an MIC value of 400 µg. However it had no impact on *Pseudomonas* and *Bacillus* cultures. When compared with the standard Nistatin, MIC of the pigment was observed as 80 µg. for *Alternaria* and for *Fusarium* MIC was observed at 160 µg. Insecticidal activity 100 % mortality was found against Cockroaches and tropical ants while 85 -71% was observed with termites and pyramid ants.

Keywords—*Serratia*, anti microbial, antifungal, insecticidal, Prodigiosin

I. INTRODUCTION

Pigment production is a unique character found only in few bacterial species. Bacteria produce pigments for various reasons, in some bacteria it is used for photosynthesis while in some it is an important virulent factor. However bacterial pigments are gaining commercial importance in various industries like food, textile, cosmetics, pharmaceutical and other industries. Bacterial pigments are advantageous over others as they are cost effective and ecofriendly. Commercially important pigments are produced biotechnologically by bacteria. Anthocyanin, Prodigiosin, violacein, Monascin, Flavin, Metacyclo prodigiosin etc. are some of the stable bacterial pigments [1,2].

Bacterial pigments are valuable secondary metabolites, which are explored as antimicrobials, biopesticides, and insecticides. Biopigments produced by plants have several limitations like insolubility and unavailability, hence microbial source becomes the only alternative for bio pigment production. The microbial pigments are of great interest due to their stability and the ease of cultivation and it

has become an interesting research field with several industrial applications.[3]

Prodigiosin is one of the secondary metabolites and bio pigment produced by many bacterial strains such as *Pseudomonas mangleslorubra*[4], *Vibrio psychroerythrous*[5], *Alteromonas rubra*, Actinomycetes such as *Streptomycespp Nocardia spp.*[6]. And *Serratia marcescens*.

The prodigiosin was first identified and characterized in *Serratia marcescens*. Prodigiosin (5((3-methoxy-5-pyrrol-2-ylidene-pyrrol-2-ylidene)-methyl)-2-methyl-3-pentyl-1Hpyrrole) is a secondary metabolite alkaloid with a unique tripyrrole chemical structure [7,8]. Prodigiosin synthesized in bifurcated pathway, in which mono and bipyrrrole precursors are synthesized separately and then coupled to form the red cell associated pigment [9]. Usually pigment producing strains are nonpathogenic when compared to the nonpigmented strains, thus reducing the risk of infection during mass production. *Serratia marcescens* produce both

intracellular and extracellular pigment during aerobic process/respiration.[10]

Prodigiosin has been reported to have many applications such as antibacterial, antifungal, tumor suppressor, antimalarial, antiprotozoal, immunosuppressive etc. due to its natural red pigmentation it can be used as a natural food colorant and can be used in textile industry. The biochemical mechanism of *Serratia* sp shows production of potentially active molecules like pyrrolnitrin, oocydin A, carbapenem, prodigiosin, cell-wall/membrane degrading enzymes like chitinase.[11] *Serratia* produces both water insoluble and water soluble pigments, like in *Serratia marcescens* red water insoluble pigment has antibiotic activity, whereas water soluble reddish-violet pigment with superoxidase dismutase has mimetic activity.

Related Work

Prodigiosin Antifungal activity was reported by Martha Ingrid in 2015 against *Mycosphaerella fijiensis* [12] and the antibacterial activity against 18 different bacterial species so far [13]. Cytotoxic and anticancer activity of prodigiosin was published by Phatake.Y.B and Dharmadhikari S.M in 2016[14]. Antimalarial activity of Prodigiosin from *Streptomyces* was reported by Estelle Marchal et al in 2014. on *Plasmodium falciparum*[15]

Applications of prodigiosin encourage one to develop a cost-effective bioprocess for its production. Multi-facilitated potency of prodigiosin pigment produced by *Serratia* could be explored not only for antibacterial and antifungal but also as effective biocontrol agent in agriculture. Present study therefore aimed to obtain a potential isolate of *Serratia* for prodigiosin production. Evaluation of the pigment activity was carried out with four bacterial strains, two fungal plant pathogens and four insect pests.

II. METHODOLOGY

Medias employed:

Nutrient Agar, Nutrient Broth, Glycerol Peptone Broth, Peanut Broth, Sesame Broth, Potato Dextrose Broth.

Microbial Cultures used in present study were *Serratia* isolate and *Serratia marcescens* YAJS for prodigiosin production.

Bacterial cultures - *E.coli*, *Staphylococcus*, *Pseudomonas* and *Bacillus* were used for antibacterial activity testing and were borrowed from veterinary college. For antifungal activity, two fungal pathogens *Alternaria*, *Fusarium* were employed.

Isolate/ Sample collection:

Sewage water sample was collected Musi River and screened for *Serratia marcescens*. The collected sample was serially diluted and plated on nutrient agar plates. Red pigmented

colonies were isolated and purified from plates after incubation at 37°C for 24 hours. Laboratory isolate *Serratia marcescens* YAJS was used in present study for comparison as it was characterized strain. Both Cultures were maintained on nutrient agar plates. Red pigmented colonies were obtained from plates after incubation at 37°C for 24 hours and preserved at 4°C.

Biochemical Characterization

The isolate was subjected to morphological and biochemical characterization by performing Gram's staining, Motility staining, Onpg test, IMViC tests, Urease test, Phenylalanine deaminase test, Sugar fermentation tests, Catalase test, Decarboxylase test, Amylase test and Hydrogen sulphide test as per standard methods

Prodigiosin production

Shake flask experiments were conducted with four different media for prodigiosin production. 50 ml of sterile broth in a 250 ml conical flask were inoculated with overnight grown culture of *Serratia* as inoculum. Conditions affecting prodigiosin production like media, temperature and incubation time were determined in shake flask experiments.

Extraction of the pigment:

Culture Broth containing the pigment was centrifuged at 6000rpm for 10 minutes. Pigment was extracted from Cell Pellet by addition of acetone, methanol or ethanol, and centrifuged at 10,000rpm for 10 minutes. Debris was removed and supernatant was carefully collected into a sterile petri dish. Plates containing the extracted pigment was left overnight at room temperature evaporate the solvent. The extracted pigment was used for estimation and for further investigation.[16]

Estimation of prodigiosin:

Spectrophotometric estimation of prodigiosin was done by using the following equation.[17]

$$\text{Prodigiosin unit/cell} = \frac{[\text{OD}_{499} - (1.381 \times \text{OD}_{620})]}{\text{OD}_{620}} \times 1000$$

Where OD_{499} - pigment absorbance

OD_{620} - bacterial cell absorbance

1.381 - constant

Purification of the pigment/ thin layer chromatography:

The pigment was purified and analyzed by thin-layer chromatography with silica gel G-60 F25~ (Merck, Mumbai, India). The solvent system consists of chloroform: methanol (95:5; v/v). The chromatography chamber with the solvent was kept for 20 min. for the equilibration. The sample was spotted on the silicagel sheet using a capillary tube and air dried. The TLC sheet was then dipped in the solvent system. After 45 minutes the TLC sheet was carefully removed and Rf was calculated.[18]

Antibacterial activity:

Antimicrobial activity of the extracted pigment was tested against common pathogens such as *Staphylococcus*, *Escherichia coli*, *Pseudomonas*, *Bacillus* by tube dilution assays. MIC was determined in test tubes by taking different concentrations of prodigiosin in nutrient broth and 100µl of test cultures of were added to each test tube. Four sets of test tubes were made to determine MIC for each culture. These tubes were incubated for 24 hours at 37°C. Uninoculated test tubes were set as control. After incubation, tubes containing the test specimen were observed for growth by turbidimetric assay. Absorbance was measured at 600nm. Minimum concentration at which growth was observed to be inhibited is considered as MIC.

Antifungal activity:

Antifungal activity was measured by a quantitative micro spectrophotometric assay as per Broekaert et al., 1990 method.[19]. Micro plate method, previously described [20], was slightly modified to determine MIC values of pigment. Pigment extract were serially diluted, ranging from 1/2 to 1/100 dilution from the crude extract in each well, 10µl of fungal spore suspension was mixed to the pigment containing 70µl of PDB. Sterile distilled water instead of test solution is used as a negative control and nystatin of an antifungal slide as positive control. The microtiter plates were incubated for 2-3 days at 27°C. Experiment was done in duplicates. MIC readings were performed before and after incubation after allowing the spores to sediment, absorbance was measured spectrophotometrically with a microplate reader at 630nm. MIC values were calculated by comparing growth in control wells and extract blank with uninoculated plates. The MIC of the extract was defined as the lowest concentration of the pigment extract causing the growth inhibition of more than 90% at 48 hours, as compared to the control.

Insecticidal activity:

The insecticidal activity was carried out on house hold pests like cockroaches, termites, pyramid ants and tropical fire ants in glass beakers by spraying pigment solution, beakers were covered and placed at room temperature. 5-10 insects were placed in each of the beaker with a control which had no pigment spray. The insecticidal effect of pigment was determined by counting the number of dead insects after 24hrs and their mortality was calculated using the formula. Insecticidal activity was calculated from the data obtained from triplicates.

The mortality rate was calculated using the formula [21]

$$\text{Mortality percent} = \frac{(X - Y) \div 100 - Y}{100} \times 100$$

X = percent mortality in treated sample.

Y = percent mortality in control.

III. RESULTS AND DISCUSSION

Present study was taken up with an aim to isolate a potential prodigiosin producer from the natural source. For this purpose, Musi river water sample was collected and plated on nutrient agar plates after serial dilution in the laboratory. Red colour colonies on the agar plate were suggesting the possibilities of the *Serratia* as shown in figure 1, however preliminary morphological and biochemical characterization was also attempted to confirm the isolate as *Serratia* species. Colony morphology of the *Serratia* was observed to be red color pigmented, rounded, raised, and opaque, with smooth surface and with entire margin was observed on Nutrient agar plate. Later the morphological and biochemical characteristics were studied by the standard methods in the laboratory and the obtained data is represented in Table no.1 *Serratia marcescens* YAJS, an laboratory strain which was earlier characterized by molecular method was also used for comparison purpose. The cultural, morphological and biochemical characteristics of the isolate from musu river sample were matching with the characterized *Serratia* strain YAJS, hence it was used for prodigiosin production.

After confirming the morphological and biochemical characteristics of *Serratia* isolate, different growth medias were prepared and were inoculated with the Culture for Pigment production. In a 250ml conical flask, 50 ml of sterile growth media such as Glycerol peptone broth, Sesame broth, Peanut broth and Nutrient broth were taken and inoculated with *Serratia* culture and incubated at 30 °C for pigment production. Production of Prodigiosin was evident in the incubated flask as it being an extracellular product, broth colour was changed to pink to dark red colour. Culture samples were used to estimate the amount of prodigiosin produced by spectrophotometric assay. And the results obtained were represented in figure 2. Among the four media tested for prodigiosin production peanut broth could support maximum production by 72 hrs of incubation. No pigment was found with glycerol broth.

In order to determine the ideal or suitable conditions for maximum production of Prodigiosin, temperature and incubation period were investigated using peanut broth. Experiments were designed by setting up the culture flask after inoculation by incubating at three different temperature 28°C, 30°C and 37°C. From the figure 4, it was understood that maximum amount of prodigiosin production was at 30°C in peanut broth than in any other media used in the study. Similarly when incubation period for prodigiosin was tested using peanut broth as growth media and incubating at 30°C, high yield was found at 72hrs of incubation as seen from the figure 5.

After understanding the process parameters which support maximum production, next the pigment was extracted from

the culture broth as per the method described earlier, as shown in figure 6, The extracted pigment purity was confirmed by the spectrophotometric method and also by TLC (data not shown).

The main aim of the present study was to evaluate the antimicrobial activity of Prodigiosin against the bacterial pathogens like *E.coli*, *Staphylococcus*, *Psuedomonas* and *Bacillus* cultures by determining MIC in tube assay. Experiments were conducted in duplicates with each test organism and the results obtained were graphically represented in Figure 7,8,9,10. Prodigiosin was observed to be effective against *E.coli* and *Staphylococcus* with an MIC value of 400 µg. However it had no impact on *Psuedomonas* and *Bacillus* cultures. M.V.Suryavanshi et al. in 2016 reported antibacterial activity of prodigiosin by agar well assay on opportunistic skin surface pathogen *Staphylococcus aureus*. [22]. However, present study found out the MIC of prodigiosin against veterinary pathogens *E.coli* and *Staphylococcus*

Antifungal activity of prodigiosin was tested on two fungal pathogens such as *Alternaria* and *Fusarium*. *Alternaria* belongs to the genus ascomycete fungi and well known as major plant pathogen. They are ubiquitous in environment and cause about 20% of agricultural spoilage [23]. *Alternaria alternata* causes early blight of potato, Leaf spot disease in *Withania somnifera* and can infect many other plants. Some of the species which causes diseases in crops are *Alternaria arborescens* causes stem canker of tomato, *Alternaria arbusi* causes leaf lesions on Asian pears, *Alternaria brassicae* infects many vegetables and roses, *Alternaria solani* causes early blight in potatoes and tomatoes.

Fusarium is a large genus of filamentous fungi and are widely distributed in soil and associated with plants. Most species are harmless saprophytes, and are relatively abundant members of the soil microbial community. Some species produce mycotoxins in cereal crops that can affect human and animal health if entered into the food chain. About 1000 species of *Fusarium* have been noted till now. *Fusarium graminearum* commonly infects barley if rainfall is delayed in the season and contamination in barely results in head blight, [24] in extreme conditions leading to pink coloration in barely. This species also causes root rot and seedling blight. *Fusarium oxysporum* causes panama disease of banana, basal rot and yellowing of leaves of daffodils.

Detection of antifungal activity was done by using microspectrophotometric assay in which microtiter plates containing the test sample and control were incubated for 48 hours. After incubation the absorbance was read by using microspectrophotometer. When compared with the standard Nistatin, MIC of the pigment was observed as 80 µg. for

Alternaria MIC for *Fusarium* was observed at 160 µg, as shown in the figure 11,12. Present study establishes the antifungal activity of Prodigiosin on two fungal pathogens which were not tested or reported earlier in literature.

Insecticidal activity of prodigiosin was carried out by spraying on to house hold pests like *Periplaneta americana* (cockroaches), *Isoptera* (termites), *Dorymyrmex insanus* (pyramid) and *Solenopsis geminata* (tropical fire ants). Termites are known to cause damage to wooden furniture and Cockroaches, cause contamination to food and indirectly responsible for food borne infections like dysentery. Insecticidal activity was determined by calculating the mortality rate for each insect pest. 100 % mortality was observed against Cockroaches and tropical ants while 85 -71% was observed with termites and pyramid ants. Presently Baygon spray, Hit and Care & Guard, Lakshman Rekha are being used to control cockroaches, however frequent use of these products are resulting in resistant varieties of cockroaches.

IV. CONCLUSION AND FUTURE SCOPE

Present study establishes the antifungal activity of Prodigiosin on two fungal pathogens which were not tested or reported earlier in literature. This study gives a scope to develop formulations based on prodigiosin as biocontrol agent against cockroaches and termites which is more cost effective and ecofriendly.

FIGURES

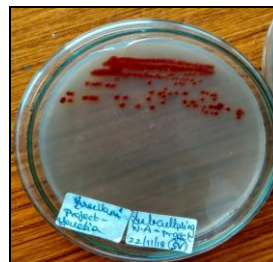


Figure1 *Serratia* on Nutrient agar plate



Figure 2: Different culture media.

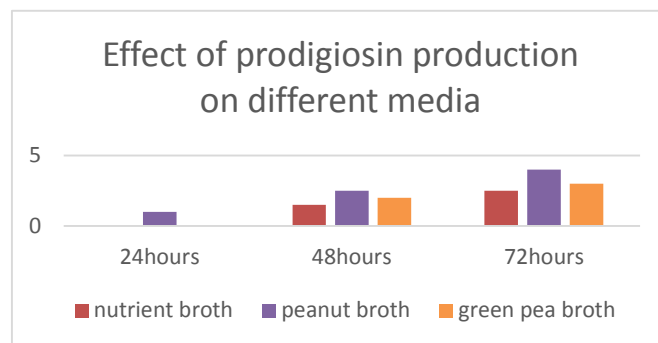


Figure 3 Effect of prodigiosin production on different media.

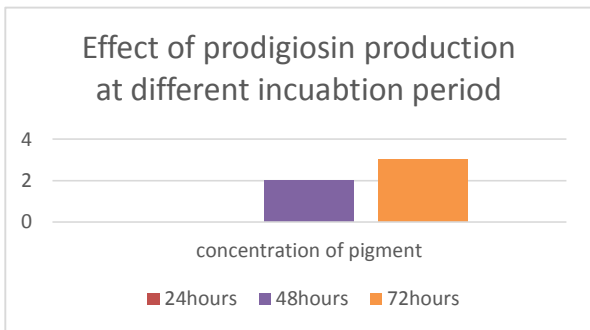


Figure 5 Effect of prodigiosin production at different incubation

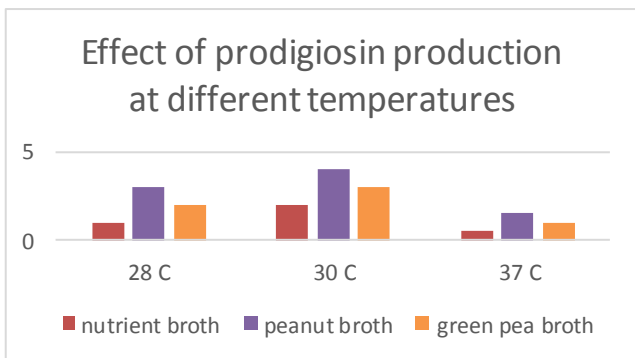


Figure 4 Effect of prodigiosin production at different temperature

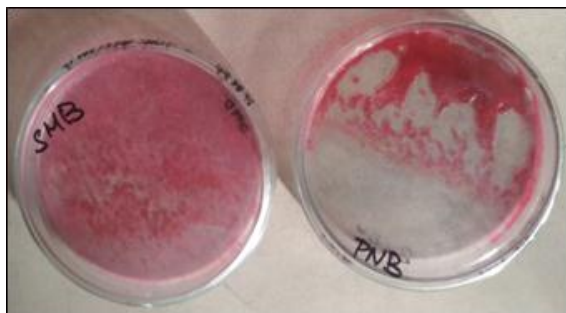


Figure 6 Pigment production on sterile petri dish

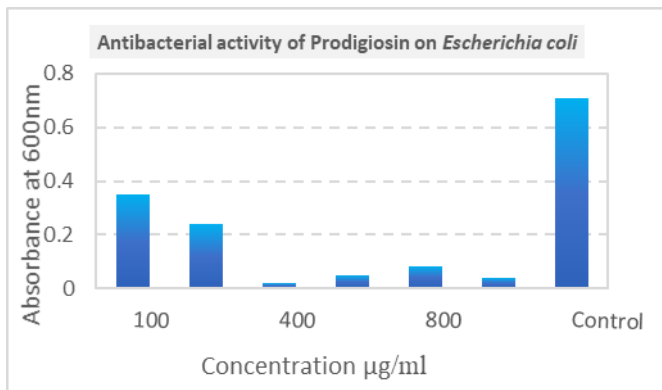


Figure 7 Antibacterial activity of prodigiosin on E.coli

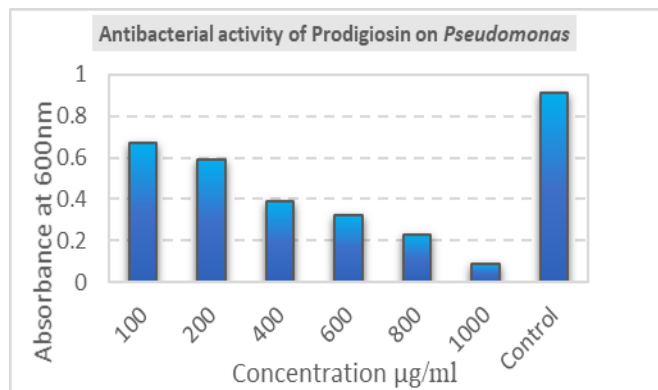


Figure 9 Antibacterial activity of prodigiosin on Pseudomonas

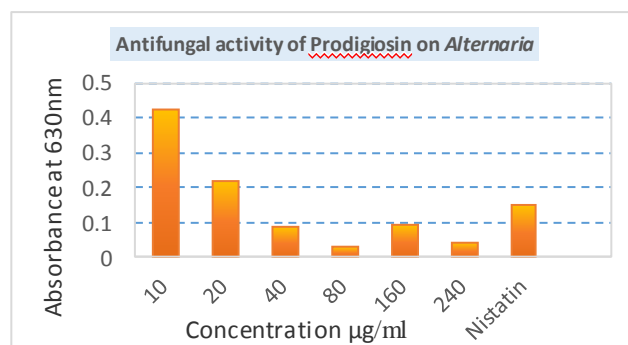


Figure 11 Antifungal activity of prodigiosin on Alternaria

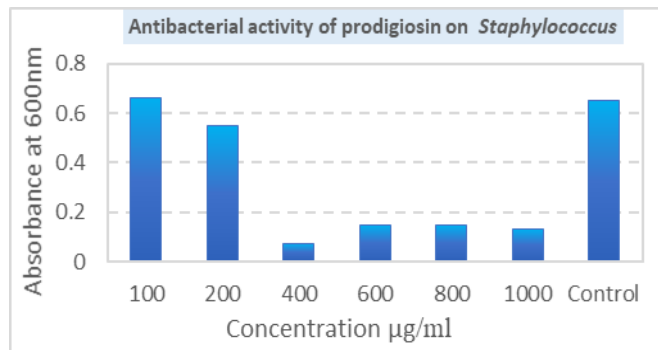


Figure 8 Antibacterial activity of prodigiosin on Staphylococcus

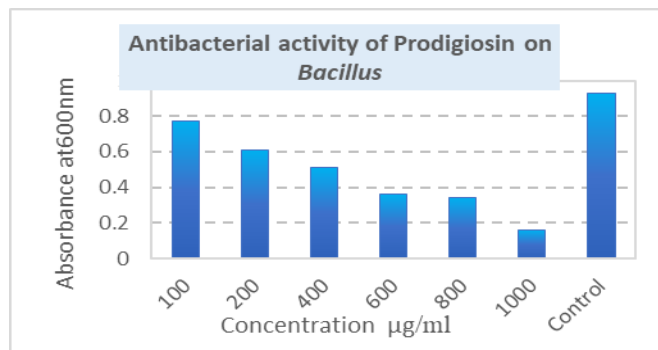


Figure 10 Antibacterial activity of prodigiosin on Bacillus

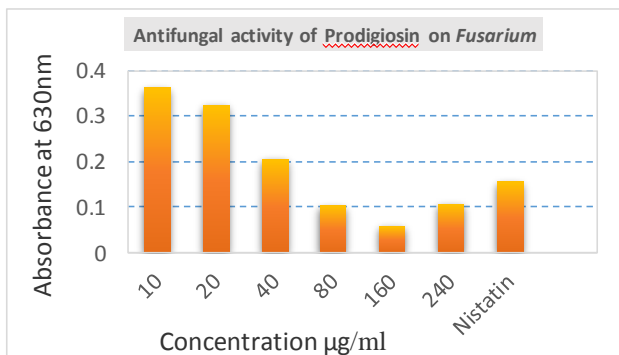


Figure 12 Antifungal activity of prodigiosin on *Fusarium*



Fig 12 Testing the insecticidal activity on Cockroaches



FIGURE 13 Testing the insecticidal activity on Fire Ants.



FIGURE 14 Testing the insecticidal activity on Termites.

TABLE 1

Identification tests	Results
Shape of the organism on Nutrient agar plate	Round, raised, pigmented with entire margin
Size of the organism on Nutrient agar plate	Small, rounded about 0.3cm
Gram's Staining	Gm negative, rod shaped bacilli
Motility by Hanging drop method	Motile
Indole test	Negative
Methyl red test	Positive
Voges Proskauer test	Negative
Citrate utilization test	Positive
Urease test	Negative
Phenyl alanine deaminase test	Negative
Decarboxylase test	Positive
Catalase test	Positive
Amylase test	Positive
Hydrogen Sulphide Test	Negative
ortho-Nitrophenyl -β-galactoside test	Positive
Sugar fermentation test - Lactose, Glucose, Sucrose, Mannitol	Positive

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