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**MICROBIOLOGY**

# Isolation, Characterization and Medium Optimization of Rhizobium Symbiont(S) From *Sesbania aculeata* (Dhaincha)

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**ABSTRACT**

*Sesbania aculeata* (*Dhaincha*) is a well known 'green manure crop' which is been widely used in organic farming as un-decomposed green manure to improve soil and crop productivity. The current study attempts to isolate and characterize (both morphologically and physiologically) Rhizobial symbionts collected from root nodules of five *desi Dhaincha* plants grown in five different regions of West Burdwan district, West Bengal, India. One isolate was chosen from each plant and they were designated as RW1-RW5. All of these five isolates were subjected to various biochemical tests and it was found that the strain RW3 was biochemically distinct from the other four isolates that put it in a different species category. All the five isolates were Gram negative; rod shaped bacterium and could ferment various carbohydrates as their carbon source. But it was to be noted that all the strains were weak fermenters for mannitol, the principal ingredient of the conventional YEM medium. Except RW3, all the other isolates were potent producers of amylase, an industrially important enzyme. The RW3 strain was the sole producer of cellulase, another enzyme of economic value. All the four isolates, excluding RW3 were obligate aerobic organisms giving positive results for both catalase and oxidase tests. The RW1 Rhizobial isolate was chosen at random for further physiological studies and it was found that its optimum temperature and pH for growth was 28 °C and & 7.0, respectively that reflected the typical features of *Rhizobiaceae* family. It was a non-halophile exhibiting maximum growth at only 1% concentration of NaCl. It was later subjected to large scale cultivation (5-10 liters of broth media) with a potential to be applied as a biofertilizer by designing a suitable broth medium instead of the normal YEM broth. 6% glucose yielded maximum biomass along with 1.5% $MgSO_4$, 2% $KH_2PO_4$, 1% NaCl and 6% yeast extract as indicated by $A_{540}$ of the inoculated medium after 48 hrs of growth. This modified medium such designed for biofertilizer production utilizing RW1 Rhizobial isolate could be considered for mass scale culture of the *Dhaincha* symbiont although field trials are recommended. Antibiotic Sensitivity Test on the RW1 strain revealed that the bacterium was most sensitive to streptomycin and kanamycin and was least affected by ampicillin as evident because of its Gram negative nature.

**Highlights**

- Five Rhizobial isolates (RW1-RW5) were isolated from five *Dhaincha* plants (*Sesbania aculeate*) form five different regions of West Burdwan district, West Bengal, India and were subjected to various biochemical tests that put the strain RW3 under a different species category owing to its distinct biochemical properties. Excluding RW3, all the four isolates were potent producers of amylase enzyme while RW3 was the only producer of cellulase enzyme. Both of these enzymes are of commercial importance.
- Excluding RW3, all the other four Rhizobial strains were obligate aerobes giving positive for both catalase and oxidase tests. RW3 was only weakly positive for Oxidase teat.
- All the strains were weak fermenters of mannitol, the conventional carbon source used in YEM medium.

- RW1, a typical Rhizobial symbiont of the desi *Dhaincha* plant grows optimally at 280C temperature, pH 7.0 and at 1% concentration of NaCl. It exhibited maximum sensitivity to streptomycin and kanamycin and was the least affected by ampicillin.
- A suitable production medium was optimized by modifying the conventional YEM medium for the large scale cultivation of the RW1 Rhizobial strain containing 6% glucose, 1.5% $MgSO_4$, 2% $KH_2PO_4$ and 6% Yeast Extract with 1% NaCl.

**Keywords:** Root nodules, nitrogen fixation, biochemical and physiological characterization, YEM medium, biofertilizer.

*Dhaincha* (*Sesbania aculeata*), a very common annual leguminous plant (shrub) is found to be widely grown as weeds in the wastelands and field bunds of dry and semi-arid regions of many states of Indian subcontinent and is one of the common and important components of green and organic manure. The leaves and twigs of the *Dhaincha* plants are used as one of the raw materials for the preparation of un-decomposed green manure in organic farming and are, therefore, considered as a 'green manure crop' that yields considerable amount of accumulated nitrogen and other nutrients like phosphorus and potassium. Hence, the shrub is of agricultural importance. Like many other leguminous plants, the root nodules of *Dhaincha* plant are also inhabited by the bacteria of *Rhizobium* species that belong to the Rhizobiaceae family, a group of symbiotic bacteria that can fix the atmospheric nitrogen to be utilized by the host plant and thus, also improves soil fertility. Rhizobium is well known as one of the most commonly used and efficient bio-fertilizers and thus plays a crucial role in agriculture. Bio-fertilizer induces plant growth, improves soil productivity and is been accepted globally as an alternative choice for chemical fertilizers as the formers are harmful for the natural eco-system because of their negative impact on the environment. Rhizobia infect the roots of the host leguminous plants and form specialized lumps called as 'root nodules' within which occurs the fixation of nitrogen, a complex biochemical process catalyzed by the bacterial nitrogenase enzyme. The soluble forms of nitrites and nitrates are incorporated by the root cells and are utilized for the synthesis of nucleic acids and proteins. In return, the host plant supplies the required nutrition, energy and shelter for the growth and multiplication of the bacteria. This symbiosis lowers the requirement of nitrogenous chemical fertilizers (Dilworth and Parker 1969; Booling *et al*. 2007; Hunter *et al*. 2007) and enhances the growth and development of not only leguminous plants but also other agriculturally important crops when applied as bio-fertilizer. The Rhizobiaceae family actually contains six different bacterial genera viz. *Rhizobium*, *Sinorhizobium*, *Mesorhizobium*, *Allorhizobium*, *Azorhizobium* and *Bradyrhizobium* (Okazaki *et al*. 2004) all of which are symbiotic nitrogen-fixers and exhibit different host specificities. But it is also true that several different rhizobial species have been isolated from the nodules of a single legume. On the basis of the growth rate, the rhizobia can be broadly classified into two major groups-fast growing rhizobia and slow growing rhizobia (Lohis and Hansen 1921). The fast growing rhizobia have an approximated generation time of less than six hours while that of slow growing rhizobia is greater than 6 hours when they are grown in selective broth media (Elkan 1992). Previous studies on isolation and characterization of Rhizobium strains isolated from root nodules of *Pisum sativum L.* (Deshwal and Chaubey 2014) have focused light on the biochemical and physiological properties of *Rhizobium leguminosarum*. Similar work done by Shahzad F. *et al*. 2012 involved the study of biochemical properties of *Rhizobium meliloti* form the root nodules of alfalfa. Like ways, isolation and characterization of Rhizobium strains form the root nodules of fenugreek have also been done (Singh B. *et al.* 2008) that gives us an idea about the rhizobial species that infects the roots of the said plant. The current study involves the isolation, characterization and medium optimization for the Rhizobium strain(s) from one of the so called 'green manure crop'-*Dhaincha* with an insight to design a suitable medium for the lab cultivation of the isolated rhizobial species that could be exploited as a biofertilizer if appropriate measures are adopted. *Dhaincha* is mostly treated as a weed as it is not of agricultural value (since it is not a crop) and so, the symbiotic rhizobia that infects the roots of this plant has not been studied in detail so far. The present study aims to explore the features of the rhizobial

strain of *Dhaincha* plant along with designing a suitable medium for its large scale cultivation with an intention to utilize it a as a biofertilizer. In this study, root nodules for the *Dhaincha* plants were collected from five different regions of West Burdwan district, West Bengal in order to find if all the rhizobial isolates exhibit similar biochemical/ physiological properties and also to investigate nutritional requirements of such an isolate for its large scale cultivation in lab.

## MATERIALS AND METHODS

**Collection of Dhaincha plant and root nodules:** Young and healthy *Dhaincha* plants were collected randomly from five different regions of West Burdwan district, West Bengal. All the plants were uprooted carefully to obtain intact roots and root nodules. The plants were transported to the laboratory facility within 2 hrs. of their collection. The fresh and plump root nodules were then detached from the roots and were subjected to surface sterilization treatment for carrying out the isolation of the nodulating bacterial flora. A total number of 30 nodules were collected from all the Dhaincha plants (i.e.6 nodules were selected from each plant) for subsequent analysis.

**Isolation of root nodulating bacteria:** The detached root nodules were washed properly under tap water to get rid of the adhering soil and dust particles. The washed root nodules were then soaked in 95% ethanol for 2-3 minutes followed by washing with 0.2% mercuric chloride solution for 2 minutes. After surface sterilization, the nodules were washed with sterile distilled water in order to remove the residual $HgCl_2$ that may remain attached with the nodules. Nodule disruption to release the intracellular bacteria may be accomplished by stabbing (Brewin *et al.* 1983), crushing (Bromfield *et al*. 1984) or by cutting (El Hassan *et al*. 1986). The crushing method was adopted for the current work in which the root nodules were transferred in clean and dry sterile test tubes and were crushed with 2 ml. of sterile distilled water using a sterile glass rod until a milky suspension was obtained. This fluid contained the nodulating Rhizobial flora. Five different test tubes, marked and labeled accordingly were used for nodules collected from Dhaincha plants grown in five different regions of the said district of West Bengal. An aliquot of 0.1 ml from each tube was then inoculated aseptically on separate pre-sterilized petri plates containing YEMA (Yeast Extract Manntiol Agar; purchased from Merck®, India) medium by Spread Plate Method inside the Laminar Air Flow Work Station. The plates were then incubated at $28^0$C temperature in an incubator for a period of 24-48 hrs. After the incubation period is over, the plates for each of the samples collected from different regions of West Burdwan district were observed for the growth of the colonies and the colony features were studied. Following the study of the colony features, the isolated colonies were subjected to Gram Staining and microscopic observation to detect the presence of Gram negative rods. One promising colony form each YEMA plate that exhibited all characteristic colony features as well as Gram nature of Rhizobium was randomly chosen and it was then transferred to YEMA slants (containing 2.0% agar; pH 7.0) in order to obtain pure culture of the selected isolate(s). The slants were stored at $4^0$C temperature for future use and also to avoid contamination.

### Biochemical Characterization

**(a) IMViC Tests:** Indole, Methyl Red, Vogues-Proskauer and Citrate Utilization Tests were performed for each of the five isolates (one from each region) according to the standard protocol (Dubey and Maheshwari 2010) and the results were tabulated. All the regents/media required for the tests were purchased either form Merck®, India or SRL®, India and were of analytical grade.

**(b) Starch Hydrolysis Test:** Starch could be hydrolyzed by amylase, an extracellular enzyme produced by many bacterial species. In order to detect the starch degrading activity of the selected Rhizobial isolates, all of them were grown in Starch Agar medium (containing 1% starch; pH 7.0) and after incubation at $28^0$C temperature for a period of 48 hrs, the plates were observed for the growth of the isolated bacteroides. The hydrolysis of starch was detected by the appearance of a clear zone on the surface of the starch agar plates around the colonies after the addition of 2-4 ml.of 0.1 N iodine solution as an indicator. (Aneja 2003) and the observation for each of the five isolates were tabulated.

**(c) Gelatinase Test:** This test is done to determine the ability of the test organism to produce gelatinase

enzyme by which it can hydrolyze gelatin and utilize it as the source of carbon and /or nitrogen for its growth. Pure cultures of the five Rhizobial isolates were grown on Gelatin Agar medium (purchased from HiMedia®, India; containing 30g/L gelatin and 15g/L agar-agar; pH 7.0) and the tubes were incubated at 28 °C for 72 hrs.. Following the incubation period, the culture tubes were subjected to low temperature treatment at $4^0$C for 30 minutes. The tubes which were positive for gelatin remains liquefied due to the production of gelatinase enzyme by the isolates while the other tubes become solidified at low temperature due to lack of gelatinase activity. Then results were then tabulated accordingly.

**(d) Urease Test:** With an aim to detect the production of urease enzyme capable of hydrolyzing urea to release ammonia by the test bacterial strains, all the five isolates were grown in five separate test tubes with Urease Test Broth (purchased from HiMedia®, containing 20g/L urea and Phenol Red as the pH indicator dye). The tubes were then incubated at 28 °C temperature for 24-48 hrs. and were observed for the color change of the broth media from yellow to red that indicates a positive reaction.

(e) **Triple Sugar Iron (TSI) Agar Test:** This test is done to assay the ability of the test bacteria to ferment three carbohydrates-glucose, lactose and sucrose. The said medium was purchased from HiMedia®, India containing 10g/L lactose, 10g/L sucrose and 1g/L glucose and Phenol Red as the pH indicator dye. After the inoculation in five different test tubes, the slants were incubated at $28^0$C temperature for a period of 24-48 hrs. A positive reaction is indicated by change of the color of the TSI agar medium at the bottom of the test tube form its original reddish-orange color to yellow color. Gas production ($CO_2$) could also be detected by the formation of bubbles or cracks in the slant. Production of $H_2S$ can also be detected by blackening of the medium. The observed result was tabulated accordingly.

**(f) Carbohydrate Fermentation Profile:** In order to assay the fermentation of various carbohydrates viz. glucose, galactose, lactose, sucrose, mannitol and fructose, the test bacterial isolates were inoculated into appropriate fermentation media containing 1% of each of the carbohydrates in sterile test tubes with Durham's tubes to detect gas production. Phenol Red was used as the pH indicator dye that turns red under acidic condition (due to formation of mixed acids) and gives a positive reaction for acid production. Formation of gas could be detected by the presence of bubbles inside the small Durham's tubes. The result was recorded after 48 hrs. of incubation of all the culture tubes at 28 °C temperature.

**(g) Catalase Test:** Catalase is an important enzyme that neutralizes the toxic hydrogen peroxide formed during oxygen metabolism in all aerobic bacterial species. The enzyme converts $H_2O_2$ into water and molecular oxygen. The test is performed simply by taking a loopful of the pure cultures of the test isolates onto separate clean and dry slides followed by addition of 4-5 drops of 30% hydrogen peroxide (purchased from Merck®, India). Occurrence of bubbles due to production of $O_2$ by the activity of catalase (effervescence) is an indicator of positive reaction. The observation for each of the five slides were recorded and tabulated.

**(h) Oxidase Test:** This test detects the presence of Cytochrome c oxidase in the bacterial respiratory chain. When a loopful of bacterial culture were mixed with tetramethyl p-phylene diamine dihydrochloride (purchased form Sigma-Aldrich®, India) on a piece of dry filter paper, the color of the dye changes to blue due to reduction of the compound by the oxidase enzyme (Benson, 1994). This color change represents a positive reaction. The results for all the five nodulating bacterial strains for this test were recorded accordingly.

**(i) Cellulase Test:** In order to find whether the bacterial isolates can produce cellulose enzyme, they were inoculated onto Carboxy-Methyl Cellulose (CMC) agar medium (purchased from HiMedia®, India) by spread plate method and the plates were incubated at 28 °C temperature for a period of 96 hrs. After the incubation period, the plates were flooded with 0.3% Congo Red Dye for 30 minutes. The stain was drained off and the plates were washed with 0.1M NaCl and were observed for the appearance of clear zones around the colonies that indicate that the isolates are cellulase producers.

## Physiological Characterization

**(a) Effect of pH on Growth:** To determine the effect of pH on the growth of the selected bacterial strains,

isolate no. RW1 was chosen randomly among the five Rhizobial strains. YEM broth media were prepared and the pH of the broth was adjusted accordingly (pH 4.0, 6.0, 7.0, 9.0 and 11.0) by using 1M NaOH and 5 N HCl. Separate Erlenmeyer flasks were used for each pH. All the flasks were incubated at 28 °C for 48 hrs. and the growth was measured spectrophotometrically (instrument purchased from Systronics, India; Model No.-AU-2603) at 540 nm wavelength. The graph was plotted by taking pH on the X-axis and absorbance on the Y-axis to depict the growth variation of the Rhizobial isolate at different pH ranges.

**(b) Effect of NaCl on Growth:** The variation in growth pattern of the RW1 bacterial strain at different concentrations of NaCl (1%, 2%, 3%, 4%, 5% and 6%), the organism was grown in different YEM broth media with respective salt concentrations in separate Erlenmeyer flasks. Following incubation at 28$^0$C for 48 hrs, the absorbance was measured at 540 nm spectrophotometrically and graph was plotted accordingly as described above.

**(c) Effect of temperature on Growth:** The effect of temperature on the growth pattern of the selected bacterium was studied by exposing the pre-inoculated YEM broth media at specified temperatures (10$^o$C, 28$^o$C, 37$^o$C, 45$^o$C, 60$^o$C and 80$^o$C) for 15 minutes followed by incubating the flasks at 28$^o$C for 48 hrs. The growth variation was then determined by measuring the absorbance at 540 nm wavelength and by drawing the respective plot as already described.

## Culture Medium Optimization for large Scale Cultivation of Rhizobial Strain

Designing a suitable culture medium to scale up cultivation of Rhizobial isolates is critical since the YEMA medium contains various compounds which is actually ideal for lab scale cultivation. The designing was done based on the growth pattern of RW1- one of the five selected isolates from *Dhaincha* root nodules chosen at random. The concentrations of the components of the YEM broth medium were varied for 5 liters of liquid culture of the selected bacterial strain in order to find out the most suitable concentration of each specific constituent for the large scale culture of the organism that might help in its application as a biofertilizer. The modifications were done as follows:

(i) Different concentrations of sucrose (2%, 4%, 6% and 8%) were used as alternative of mannitol keeping all other components unchanged as compared to the normal YEM broth medium. Mannitol was substituted since earlier experiment showed that all the isolates were weak mannitol fermenters.

(ii) Various concentrations of glucose (2%, 4%, 6% and 8%) were used as a replacement of sucrose in order to see if there is any affect on the growth rate by keeping all the other ingredients of the YEM broth same.

(iii) The concentrations of $MgSO_4$ were varied (0.5%, 1%, 1.5%, 2%, 2.5%) to detect change in the growth pattern, if any. All other ingredients were same as in the normal YEM broth.

(iv) $MgSO_4$ was replaced by $FeSO_4$ of equal concentrations like that of $MgSO_4$ keeping the other ingredients unchanged to see if $FeSO_4$ could be a suitable substituent for $MgSO_4$.

(v) The concentrations of $KH_2PO4$ were varied (0.5%, 1%, 1.5%, 2%, 2.5%) to see change in the growth pattern, if any. All other ingredients were same as in the normal YEM broth.

(vi) $KH_2PO_4$ was replaced by $NaH_2PO_4$ of varying concentrations (0.5%, 1%, 1.5%, 2%, 2.5%) keeping the other ingredients unchanged.

(vii) The Yeast Extract was also used at varying concentrations (2%,4%, 6% and 8%) keeping the other constituents of the normal YEM broth same.

All the ingredients for the medium formulation were purchased either from SRL® India or from Merck®, India. In each case, the growth was measured by monitoring the absorbance of the YEM broth medium (pH 6.8±0.2) spectrophotometrically at 540 nm wavelength after inoculation of the media with the pure culture of the RW1 strain and incubating at 28 $^o$C for 48 hrs.

**Antibiotic Sensitivity Test:** The sensitivity of the RW1 strain against selected antibiotics (Streptomycin, Tetracyclin, Ampicillin, Ciprofloxacin and Kanamycin) was measured by Kirbey-Bauer Method (Disc-diffusion method). This was performed by spreading 0.5ml of pure broth culture

of the isolate on the surface of the YEMA plates and then by placing the commercially available discs of the said antibiotics (purchased form Bio-Rad®, India; disc content 30 μg of the specific antibiotic $disc^{-1}$) on the inoculated plates. All the plates were then incubated at 37 °C temperature for 48 hrs. The sensitivity was measured in terms of diameter of the inhibition zones, i.e. the clear zones that appear around the colonies due to the activity of the antibiotics.

## RESULTS AND DISCUSSION

### Collection of root nodules of *Sesbania* sp. from different locations of West Burdwan

The root nodules were collected from Dhaincha plants from five different locations of West Burdwan district, West Bengal and 6 fresh nodules were selected from each plant and the samples were numbered accordingly as shown in Table 1.

**Table 1:** Collection of *Dhaincha* plant root nodules form different regions of West Burdwan District, West Bengal

| Sample No. | Region | No.of nodules collected from each plant | No.of Positive Samples |
|---|---|---|---|
| RW1 | Kulti | 6 | 6 |
| RW2 | Asansol | 6 | 6 |
| RW3 | Raniganj | 6 | 6 |
| RW4 | Rajbandh | 6 | 6 |
| RW5 | Panagarh | 6 | 5 |

**Study of colony features and Gram character:** The colony characteristics and Gram natures of the 5 chosen bacterial isolates (one from each plant sample) were recorded. The result is tabulated in Table 2. From the table, it was found that all the chosen isolates were Gram negative rod shaped bacteria which is the typical feature of the Rhizobiaceae family as mentioned in the *Bergey's Manual of Bacteriology*. The colony characteristics of the four of the isolates were almost identical excepting subtle differences in the colony color or shape which indicated that the strains might belong to the same species. Only a single isolate, RW3 exhibited quite different morphological features that might place it under a different species in comparison to the other Rhizobial strains.

**Biochemical Characterization-a) IMViC tests:** The results of IMViC tests performed for all the five Rhizobial strains (Table 3) indicated that all the selected isolates were negative for Indole,Vogues-Proskauer and Citrate Utilization tests but exhibited positive results for Methyl Red Test reflecting their capability to ferment glucose as the carbon source and produce mixed acids that lead to the change of the color of the medium from yellow to red. Many species of Rhizobium like *R.leguminosarum* gives positive for Methyl Red Test (https://modmedmicrobes.wikispaces.com/R.+Leguminosarum) although the degree of glucose utilization varied among the isolates.

**(b) Starch Hydrolysis Test:** From the observations for starch hydrolysis test (Table 3), it could be inferred that all the isolates were positive for the production of the enzyme amylase that hydrolyses starch. Excepting the isolate RW3, all the strains were strong producers of amylase indicating they might belong to the same species of Rhizobium.

**(c) Gelatinase Test:** The observations for gelatinase test (Table 3) inferred that all the isolates except RW3, showed negative result for gelatinase production that again put them under the same species category RW3 being the only exception which could hydrolyze gelatin.

**Table 2:** Study of colony features and Gram Character

| Isolate No. | Colony Shape | Colony Size | Colony Color | Opacity | Margin | Elevation | Consistency | Gram Character & Shape of Bacterium |
|---|---|---|---|---|---|---|---|---|
| RW1 | Circular | 0.4cm | White | Translucent | Entire | Raised | Mucoidal | Gram negative rod shaped |
| RW2 | Circular | 0.6cm | White | Translucent | Entire | Raised | Mucoidal | Gram negative rod shaped |
| RW3 | Punctiform | 0.1cm | Off-White | Translucent | Entire | Convex | Mucoidal | Gram negative rod shaped |
| RW4 | Circular | 0.8cm | Off-white | Translucent | Entire | Raised | Mucoidal | Gram negative rod shaped |
| RW5 | Circular | 0.4cm | White | Translucent | Entire | Raised | Mucoidal | Gram negative rod shaped |

**Table 3:** Results of IMViC Tests, Starch Hydrolysis Test, Gelatin Liquefaction Test, Urease Test and TSI Agar Test

| Isolate No. | Indole Test | Methyl Red Test | V-P Test | Citrate Utilization Test | Starch Hydrolysis | Gelatin Liquefaction | Urease Production | TSI Agar Test |
|---|---|---|---|---|---|---|---|---|
| RW1 | -- | ++ | -- | -- | ++ | -- | -- | Yellow slant, yellow butt, no $H_2S$ |
| RW2 | -- | ++ | -- | -- | ++ | -- | -- | Yellow slant, yellow butt, no $H_2S$ |
| RW3 | -- | + | -- | -- | + | + | + | Red slant, yellow butt with little $H_2S$ |
| RW4 | -- | ++ | -- | -- | ++ | -- | -- | Yellow slant, yellow butt, no $H_2S$ |
| RW5 | -- | + | -- | -- | ++ | -- | -- | Yellow slant, yellow butt, no $H_2S$ |

*++ indicates strongly positive, + indicates weakly positive, -- indicates negative result.*

**(d) Urease Test:** The results for urease test (Table 3) exhibited negative for the production of urease enzyme for all the four isolates except RW3 which was the only bacterium capable of hydrolyzing urea into ammonia.

**(e) Triple Sugar Iron (TSI) Agar Test:** The documentation for TSI agar test as displayed in Table 3 represented the fact that the inoculants-RW1, RW2, RW4 and RW5 had fermented lactose and sucrose. Because these two sugars are present in high concentration in the TSI agar medium, the acid produced by the sugar fermentation had retained in both the slant and the butt portions of the culture tubes and thus lowering the pH that led to the yellow coloration. But the isolate no.RW3 had fermented only glucose and not the other two sugars. Since the concentration of glucose in the medium is the least, acid produced was retained only in the butt while the slant portion remained alkaline because of the utilization of peptones that made the pH of the slant alkaline as evident by its red coloration. The TSI agar test again indicates that the RW3 isolate is different from the other four test rhizobial strains.

**(f) Carbohydrate Fermentation Profile:** The carbohydrate fermentation profiles of the five organisms for the 6 different sugars (Table 4) implied that the isolate RW3 could utilize only glucose, galactose and mannitol as their carbon source and produce both acid and gas although gas production was comparatively lesser in case of mannitol. The other four Rhizobial strains could ferment glucose, galactose, lactose, sucrose and mannitol producing both acid and gas while the production of gas by mannitol fermentation was also to a lesser extent like that of the RW3. None of the organisms was able to ferment fructose.

**Table 4:** Carbohydrate Fermentation Profile

| Isolate No. | Glucose | Galactose | Lactose | Sucrose | Fructose | Mannitol |
|---|---|---|---|---|---|---|
| RW1 | ++ | ++ | ++ | ++ | -- | + |
| RW2 | ++ | ++ | ++ | ++ | -- | + |
| RW3 | ++ | ++ | -- | -- | -- | + |
| RW4 | ++ | ++ | ++ | ++ | -- | + |
| RW5 | ++ | ++ | ++ | ++ | -- | + |

*++ indicates strongly positive, + indicates weakly positive, -- indicates negative result.*

**(g) Catalase Test:** The results for catalase test (Table 5) suggested that all of the five isolates are strong producers of catalase enzyme implying active oxygen metabolism in their cells and subsequent neutralization of the toxic $H_2O_2$ by the said enzyme.

**Table 5:** Results of Catalase, Oxidase and Cellulase Tests

| Isolate No. | Catalase Test | Oxidase Test | Cellulase Test |
|---|---|---|---|
| RW1 | ++ | ++ | + |
| RW2 | ++ | ++ | + |
| RW3 | ++ | + | -- |
| RW4 | ++ | ++ | + |
| RW5 | ++ | ++ | + |

*++ indicates strongly positive, + indicates weakly positive, -- indicates negative result.*

**(h) Oxidase Test:** The observations for oxidase test were tabulated in Table 5. This result provides a hint that all the four isolates apart from the exceptional RW3 were obligate aerobic in nature as indicated by the presence of Cytochrome c oxidase in their cells. The bacterium RW3 was only weakly positive for oxidase production indicating the fact that it might be a facultative aerobe in nature.

**(i) Cellulase Test:** According to the results for cellulase test, RW3 could not produce any cellulase enzyme while the other four isolates were only weak producers for the enzyme since only partial degradation of cellulose was detected as evidenced by the appearance of a faint clear zone around the colonies after 4 days of incubation. It could also be concluded that the organisms may be slow degraders of cellulose and may require more time for complete cellulose decomposition.

## Physiological Characterization

**(a) Effect of pH on Growth:** The result for pH variation assay on the growth of the selected Rhizobial isolate RW1 was depicted in Fig. 1. It shows that the bacterium is a neutrophile as the highest growth was obtained at pH 7.0. The growth was declined at higher and lower pH ranges.

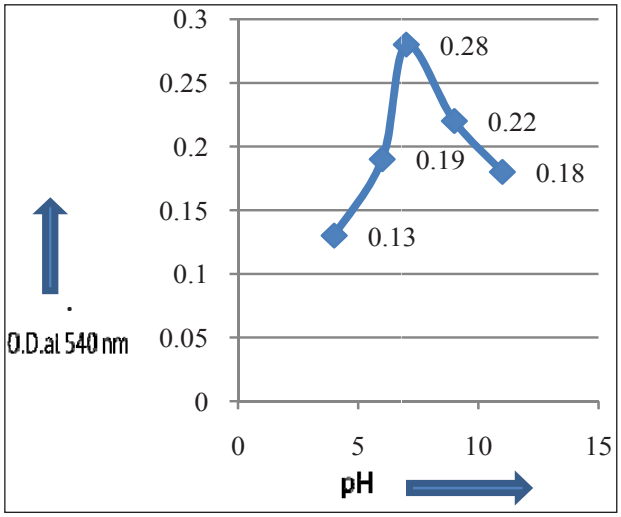


**Fig. 1:** Effect of pH variation on the growth of Isolate No.RW1.

**(b) Effect of NaCl on Growth:** From the plot (Fig. 2), it could be interpreted that RW1 isolate grows best at lowest (1%) concentration of NaCl. Salt concentrations higher than this inhibits its growth indicating that the bacterium is a non-halophilic and non-osmophilic strain of Rhizobium.

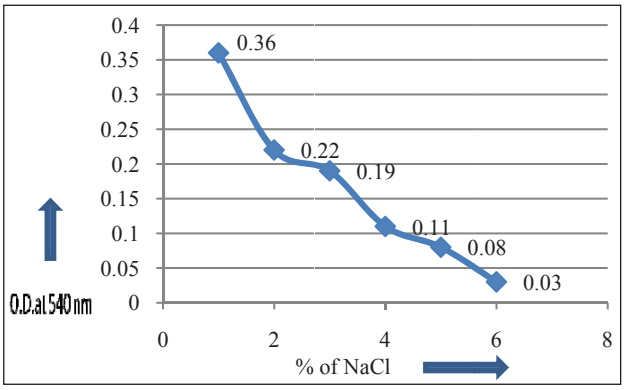


**Fig. 2:** Effect of NaCl variation on the growth of Isolate No. RW1

**(c) Effect of temperature on Growth:** The plot (Fig. 3) obtained for temperature variation assay on the growth of the Rhizobial isolate RW1 depicted that the organism is a mesophile and its optimum temperature for growth is 28 °C which is a typical feature of the family Rhizobiaceae.

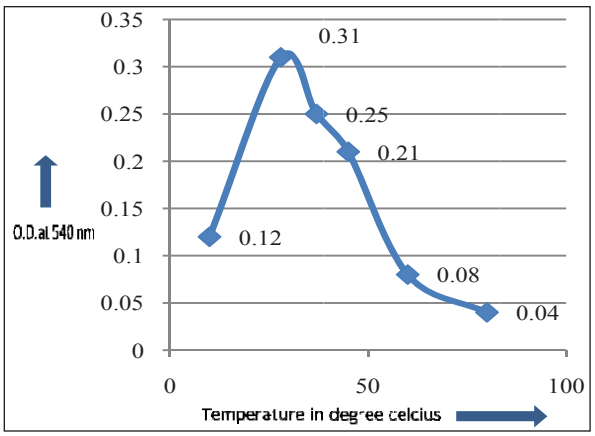


**Fig. 3:** Effect of temperature variation on the growth of Isolate No. RW1

## Culture Medium Optimization for large Scale Cultivation of Rhizobial Strain

The isolate RW1 was chosen randomly to formulate a suitable broth medium for the scale up process for mass production of this Rhizobial strain. The influence of different concentrations of different nutritional components of the YEM broth medium on the growth pattern of RW1 isolate are illustrated in the plot(s)- Fig. 4 (for sucrose), Fig. 5 (for glucose), Fig. 6 (for $MgSO_4$), Fig. 7 (for $FeSO_4$), Fig. 8 (for $KH_2PO_4$), Fig. 9 (for $NaH_2PO_4$) and Fig. 10 (for yeast extract). It was observed that in comparison to sucrose, glucose gave a better biomass yield by the bacterium at equal concentrations. Glucose

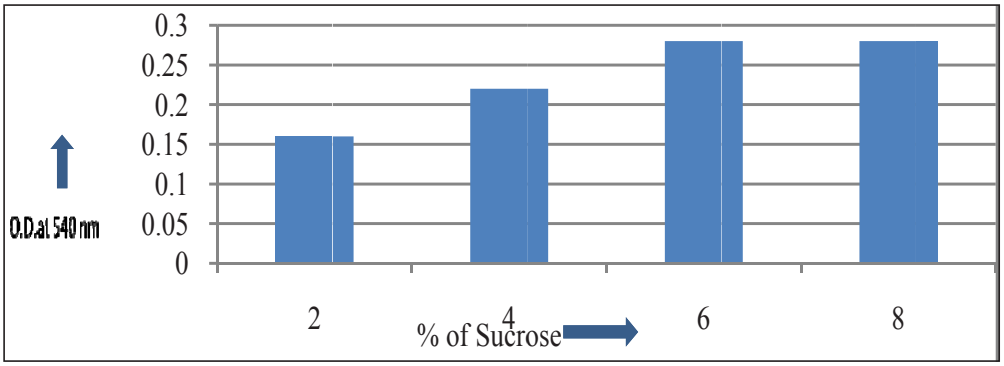


**Fig. 4:** Effect of variations of sucrose concentration on the growth of RW1 Rhizobial isolate

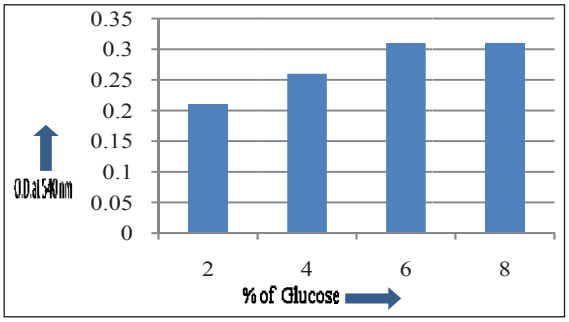


**Fig. 5:** Effect of variations of glucose concentration (as substitute of sucrose) on the growth of RW1 Rhizobial isolate

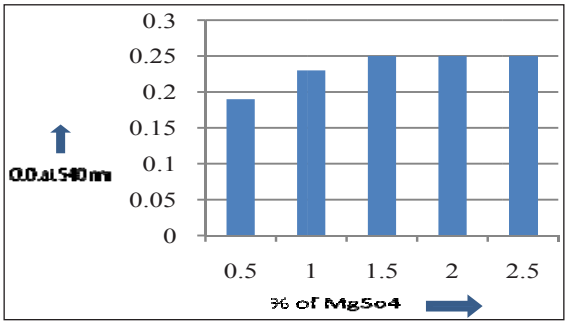


**Fig. 6:** Effect of varying concentrations of $MgSO_4$ on the growth of RW1 Rhizobial isolate

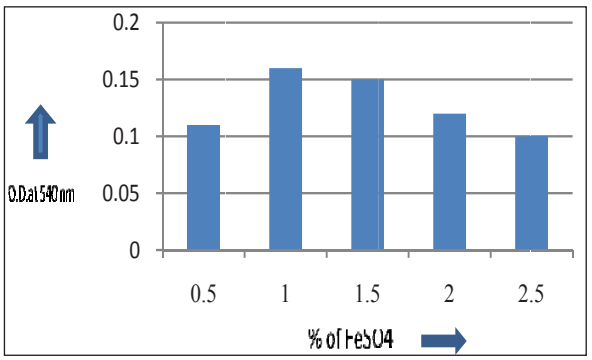


**Fig. 7:** Effect of different concentrations of $FeSO_4$ on the growth of RW1 Rhizobial isolate

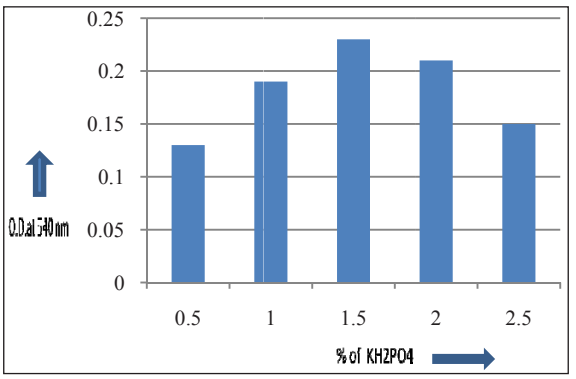


**Fig. 8:** Effect of different concentrations of $KH_2PO_4$ on the growth of RW1 Rhizobial isolate

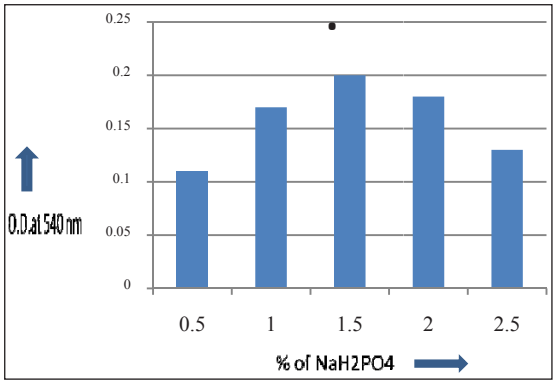


**Fig. 9:** Effect of different concentrations of $NaH_2PO_4$ (as a possible substituent of $KH_2PO_4$) on the growth of RW1 Rhizobial isolate

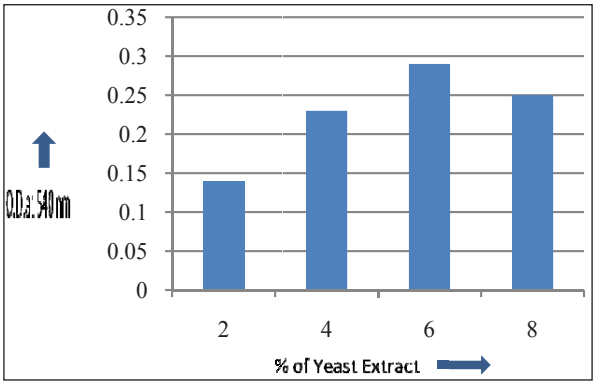


**Fig. 10:** Effect of variable concentrations of Yeast Extract on the growth of RW1 Rhizobial isolate

being a monosaccharide is easily metabolized by the organism and produced better yield than sucrose which is a disaccharide and so, is metabolized slowly compared to glucose. Glucose is also cheaper and so, it could be used as an effective carbon source in place of sucrose/mannitol for mass culture of the Rhizobial strain. 6% of glucose was found to be ideal to obtain maximum growth of the RW1 strain.

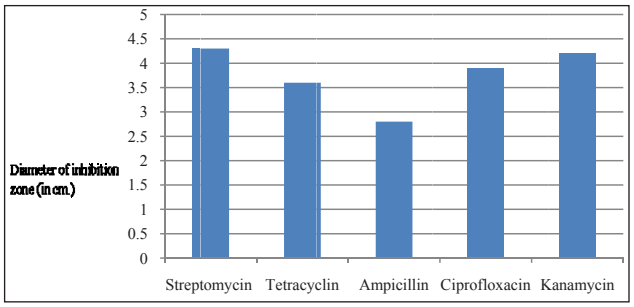


**Fig. 11:** Results of Antibiotic Sensitivity Test on RW1 strain of Rhizobium

On the other hand, when $MgSO_4$ was replaced with equal amounts of $FeSO_4$, the biomass yield was reduced to some extent although the sulfate salt of iron yielded maximum growth at 1% concentration while $MgSO_4$ gave optimum growth at 1.5% concentration. Thus, as far as biomass yield is considered, $MgSO_4$ remains the better choice but if the amount consumed is considered, then $FeSO_4$ could also be used for the mass cultivation of the RW1 strain as an alternative of $MgSO_4$. In case of potassium and sodium salts of hydrogen phosphates, both of them induced maximum growth at 2% concentration, but as total biomass is considered, $KH_2PO_4$ gave a better result as evident by higher absorbance at the same concentration and wavelength. Hence, $KH_2PO_4$ remains the phosphate salt of choice for large scale cultivation of the organism under study. As far as yeast extract as nitrogen source is examined, 6% concentration of the same yielded maximum growth. Thus, yeast extract seems to be a suitable nitrogen source of the cultivation of the RW1 strain of the *Dhaincha* symbiont.

Compiling all the data obtained from these experiments, the most suitable medium composition (producing maximum biomass yield) for the mass cultivation of the RW1 Rhizobial strain isolated from *Dhaincha* plant would be as follows:

| Component | Amount (g/L) |
|---|---|
| Glucose | 60 |
| $MgSO_4$ | 15 |
| $K_2HPO_4$ | 20 |
| Yeast Extract | 60 |
| NaCl | 10 |
| Final pH at 28-30 ˚C | 6.8±0.2 |

**Antibiotic Sensitivity Test:** The Antibiotic Sensitivity Assay revealed that the RW1 Rhizobial symbiont of the *Dhaincha* plant was most sensitive to streptomycin and kanamycin both of which are inhibitors of bacterial protein synthesis. In consistent with the fact that the organism is a Gram negative one, it was showed be least sensitive to ampicillin, an antibiotic that inhibits bacterial growth by interfering with the peptidoglycan biosynthesis and Gram negative bacteria have a very thin layer of peptidoglycan making ampicillin less effective.

## CONCLUSION

Five Rhizobial symbionts obtained from *Sesbania aculeata* (*Dhaincha*)-a 'green manure crop' grown in five different regions of West Burdwan District, West Bengal were isolated and subjected to extensive morphological, biochemical and physiological studies in the present work. Four of these isolates seemed to belong to a single species basis of their identical biochemical and morphological properties while the fifth one; designated as RW3 was found to contain distinct morphological and biochemical properties based on which it was thought to belong to a different species category. The RW3 symbiont was obtained from *Sesbania* grown in Raniganj region of West Bengal. All the isolates were found to be good producers of amylase, one of the most industrially important enzymes in present day biotechnology that occupies about 25% of the global enzyme market (Rao *et al*. 1998). RW3 isolate was the sole producer of cellulase, another such economically important enzyme while the other four strains could not hydrolyse cellulose at all. All of the isolates could ferment various carbohydrates as their carbon source fructose being the only exception. In addition, one of these isolates, RW1 was chosen randomly to formulate a suitable liquid medium for its large scale cultivation that could be utilized as a biofertilizer. The medium was designed optimally by adjusting its different nutritional constituents in order to achieve maximum biomass yield that

makes it useful for commercial application although field trials on crops are highly recommended. Further identification of the RW1 strain, a potential candidate for biofertilizer up to species level needs to be done by compiling rRNA sequencing data along with the other experimental data obtained in the current course of study. The study also indicated that the RW1 strain under investigation was most sensitive to streptomycin/kanamycin and was least effected by ampicillin that confirms its Gram negative character.

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