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Isolation, characterization and identification of *Gluconacetobacter* to develop it as a biofertilizer for sugarcane crop in Chhattisgarh

Narayan Prasad Verma and Tapas Chowdhury

Abstract
Sugarcane is highly nitrogen requiring crop and about 250 kg /ha is required for its successful cultivation. At present no suitable biofertilizer for sugarcane crop is available in Chhattisgarh, so alternative ways were searched to reduce the use of chemical fertilizers in sugarcane cultivation. To fulfill the above target for reduction of fertilizer requirement for the above crop efforts have been made to search effective bioagent which can provide nitrogen nutrition to crop under low input technology. Looking to the above facts the organism "*Gluconacetobacter"* was taken as a test organism for sugarcane as the organism has the potential to fix 150 kg atmospheric N/ha. In this connection endophytic dizotrophic bacteria were isolated from plant samples of sugarcane crop grown in different areas of kabirdham district of Chhattisgarh. Forty isolates of *Gluconacetobacter* were isolated from sugarcane juice inoculated in LGI medium. Theses isolated were found effective to fix atmospheric nitrogen and solubilize insoluble phosphates. Out of 40 isolates collected four were found very effective for above parameters. Further these isolates were characterized and identified as *Gluconacetobacter*. These isolates were also found good capacity to produce different plant growth promoting phytohormones. At present the above effective isolates are being tested for their N-fixing capacity in farmer's fields. The results are found very encouraging.

Keywords: *Gluconacetobacter,* biological nitrogen fixation, sugarcane

Introduction
Sugarcane (*Saccharum officinarum* L.) is one of the most important agricultural products for export and domestic consumption in most tropical countries. In India, farmers cultivate sugarcane in the form of cash crops. The area under sugarcane crop in India has increased by 43.87 lakh hectares to 46.71 lakh hectares during years 2016-17 to 2017-18 (Min.of Agri., GOI, 2017). In Chhattisgarh state the scope of sugarcane is bright because the area under this crop increasing in every year and now the area under this crop reached from 0.32 lakh hectares to 0.35 lakh hectares during year 2015-16 to 2016-17 (C.G. Dept. of Agri., 2017). In Chhattisgarh four sugar mills in the state are established in Kabirdham, Balod and Surajpur districts. With the establishment of these factories, there has been an increase in the area and production of sugarcane in the state and the growth in the area of sugar cane in this current year is about 7-8 % over precious years. Department of Agriculture said that Chhattisgarh has traditionally been the productive state of paddy. Now the farmers are turning towards oilseeds, pulses and sugarcane. Sugarcane is highly nitrogen requiring crop. About 250 kg Nitrogen ha^{-1} is required for its successful cultivation. Looking to the high cost of chemical fertilizers the poor and marginal farmers of the state are unable to provide recommended dose of chemical fertilizers to the crop, resulting poor productivity of sugarcane. Another demerits of chemical nitrogenous fertilizer & are the environmental problems of nitrate pollution of waters and the environment with nitrous oxide and ammonia due to long term fertilizer application which also lead for Global warming. In addition, chemical fertilizers can drain into ground water, streams, and rivers, risking human and animal health. So we have to look alternative ways to reduce the use of chemical fertilizers in sugarcane cultivation. At present no suitable bio-fertilizer for the sugarcane production is available in Chhattisgarh. As per Government of India directives farmers' income has to be doubled by 2022. Considering to the above target to reduce the fertilizer dependency for crop production it is mandatory to adopt low input technology. *Gluconacetobacter* an endophytic bacteria can supplement 150 N kg ha^{-1}, so it can be used as bio-fertilizer to fulfill the nutritional need of the crop but efforts are requiring to search such effective indigenous *Gluconacetobacter* which strain can perform better in Chhattisgarh agro-climatic conditions. Hence present study was conducted to isolate, characterize and identify an *Gluconacetobacter* isolate to develop it as a biofertilizer for sugarcane crop in Chhattisgarh.

Materials and methods

Present study was isolation, characterization and identification of *Gluconacetobacter* to develop it as a biofertilizer for sugarcane crop in Chhattisgarh. The study was conducted at the Department of Agricultural Microbiology, Indira Gandhi Agriculture University, Raipur (Chhattisgarh) during 2016-17.

Samples and sampling

Different sugarcane growing areas were identified in Kabirdham district to take samples of sugarcane plants and rhizosphere. The details of location of sugarcane associated samples are tabulated in Table 1. The samples of sugarcane (*Saccharum officinarum*) and its rhizosphere were collected in sterile bags.

Table 1: Location from where sugarcane samples were collected to isolate indigenous *Acetobater* isolates.

Sample no.	Sample name	Location of sample collection	District
1	DKWD-1	Doojari (Sugarcane)	Kabirdham
2	UKWD-2	Uslapur (Sugarcane)	Kabirdham
3	GKWD-3	Gadai (Sugarcane)	Kabirdham
4	JKWD-7	Gadai (Sugarcane)	Kabirdham
5	KKWD-9	Kusumghta (Sugarcane)	Kabirdham
6	SKWD-15	Sukhatal (Sugarcane)	Kabirdham
7	KKWD-16	Kusumghta (Sugarcane)	Kabirdham
8	KKWD-18	Kusumghta (Sugarcane)	Kabirdham
9	SKWD-19	Sukhatal (Sugarcane)	Kabirdham
10	UKWD-20	Uslapur (Sugarcane)	Kabirdham
11	SKWD-21	Saranpur (Sugarcane)	Kabirdham
12	NKWD-22	Naudih (Sgarcane)	Kabirdham
13	BKWD-24	Bagharra (Sgarcane)	Kabirdham
14	RKWD-26	Rivapar (Sgarcane)	Kabirdham
15	GKWD-28	Gadai (Sugarcane)	Kabirdham
16	UKWD-30	Uslapur (Sugarcane)	Kabirdham
17	BKWD-33	Baiharsari (Sugarcane)	Kabirdham
18	GKWD-34	Gadai (Sugarcane)	Kabirdham
19	BDKWD-37	Bdhaikunda (Sugarcane)	Kabirdham
20	JKWD-39	Jarti (Sugarcane)	Kabirdham
21	UKWD-40	Uslapur (Sugarcane)	Kabirdham
22	JKWD-41	Jarti (Sugarcane)	Kabirdham
23	PKWD-43	Pondi (Sugarcane)	Kabirdham
24	UKWD-46	Uslapur (Sugarcane)	Kabirdham
25	JKWD-49	Jarti (Sugarcane)	Kabirdham
26	BGKWD-51	Bagharra (Sgarcane)	Kabirdham
27	NUKWD-52	Naudih (Sgarcane)	Kabirdham
28	BKWD-57	Baiharsari (Sugarcane)	Kabirdham
29	UKWD-60	Uslapur (Sugarcane)	Kabirdham
30	UKWD-61	Uslapur (Sugarcane)	Kabirdham
31	JKWD-62	Jarti (Sugarcane)	Kabirdham
32	NUKWD-64	Naudih (Sgarcane)	Kabirdham
33	SRKWD-68	Saranpur (Sugarcane)	Kabirdham
34	SIKWD-69	Silhati (Sugarcane)	Kabirdham
35	RIKWD-71	Rivapar (Sgarcane)	Kabirdham
36	JKWD-74	Jarti (Sugarcane)	Kabirdham
37	PKWD79	Pondi (Sugarcane)	Kabirdham
38	UBKWD-13	Uslapur (Sugarcane)	Kabirdham
39	BGKWD-25	Bagharra (Sgarcane)	Kabirdham
40	SKWD-48	Sukhatal (Sugarcane)	Kabirdham

Isolation of *Gluconacetobacter*

Collected samples of young shoots with rhizosphere were taken from one year old sugarcane plant. Plant samples were carefully washed with tap water and surface sterilized. The semisolid LGI media: K_2HPO_4 0.2g; KH_2PO_4 0.6g; $MgSO_2.7H_2O$ 0.2g; $CaCl_2.2H_2O$ 0.02g; $NaMoO_4.2H_2O$ 0.002g; $FeCl_3.6H_2O$ 0.01g; yeast extract 0.02g; 5 ml of bromothymole blue (BTB) 5% solution in 0.2 N KOH; crystallized sucrose 100g; agar 1.8g/1 (adjusted to pH 5.5-6.0) was taken for isolation of *Gluconacetobacter* isolates. The methodology for isolation was followed as given by Cavalcante and Dobereiner (1988). The inoculated tubes were then incubated for 5-7 days at 30 ^{0}C. The growth of *Gluconacetobacter* was confirmed by appearance of enriched growth in the form of subsurface yellow and orange pellicle (Fig 1). To get pure culture the bacterial growth was streaked on plates of LGI agar medium and incubated for 6-7 days at 30 ^{0}C. After proper growth pure colony of each isolate was subcultured and preserved.

Study of phenotypic and biochemical properties of *Gluconacetobacter* isolates

The pure isolates developed on LGI agar medium were tested for the following morphological properties like colony morphology, colony size; colony pigmentation, gram reaction and shape of cell were examined. Pigmentation was assessed to grow the isolates on GYC medium (Madhaiyan *et al*. 2004) [5]. Gram staining of the isolates was examined as per Rangaswami and Bagyaraj (1993). Growth characteristics of different isolates under different temperatures (28 °C, 37 °C and 40 °C) and pH (3.5, 5.5, 7.0 and 8.0) (Kadere *et al*. 2008) [3] were also observed.

Different biochemical tests were performed to characterize bacterial isolates i.e. catalase test, gelatin hydrolysis test, motility test, ketogenesis test, nitrate reduction test, H_2S production test and IMViC test which includes indole production, methyl red test, citrate utilization test and voges Proskauer test as described by Seeley and Vandemark (1981).

Testing the Nitrogen fixation capacity of collected *Gluconacetobacter*

The ability of collected *Gluconacetobacter* isolates to fix atmospheric N_2 in culture medium was tested in liquid LGI medium by the micro-Kjeldahl method (Reis *et al.*, 1994) [7].

Results

Nitrogen fixation capacity

The present research was conducted for isolation, identification and characterization of *Gluconacetobacter* isolates. Forty isolates were isolated from stem juice and rhizospheric samples. These samples were tested for their atmospheric N_2 fixation capacities. On the basis of N-fixing capacity of collected isolates top four isolates were selected for their further characterization. The N-fixing capacity of top four *Gluconacetobacter* isolates ranging from 20.792 to 24.411µgN/mg Carbon. Similar type of N-fixing capacity of *Gluconacetobacter* isolates was also reported by Hema *et al.*, 2017 (Table 2).

Table 2: Nitrogen fixation ability of *Gluconacetobacter strain* isolates by Microkjeldhal method.

S. No	Isolates Code	µg of Nitrogen/ mg of Carbon
1	JKWD-49	20.79
2	UKWD-40	22.43
3	JKWD-74	24.41
4	KKWD-16	21.77

Morphological characterization and growth study

The top performing isolates were characterized by their cell morphology under microscope, sensitivity test at different

temperature and pH levels. They were further tested for their reaction towards different biochemical tests and gram reaction. All the tested isolates were found gram –ve in gram reaction. Their cell morphology ranged from rod to ellipsoid. The isolates showed good growth at 30 ^{0}C. All the top performing isolates showed good growth at pH 7.0 except isolate JKWD-74, which showed profuse growth even at pH 8.0 (Table-3)

Table 3: Selective phenotypic properties of *Gluconacetobacter strain* isolates.

Isolates		Morphological Characteristics		Growth Characteristics						
		Gram reaction	Cell shape	Growth on Different temperature			Growth on Different ph			
				28 ^{0}C	30 ^{0}C	37 ^{0}C	3.5	5.5	7.0	8.0
1	JKWD-49	-ve	Ellipsoid to rod	+	++	+	-	+	+	-
2	UKWD-40	-ve	rod	+	++	+	-	+	+	-
3	JKWD-74	-ve	Ellipsoid rod	+	++	+	-	+	+	+
4	KKWD-16	-ve	rod	+	++	+	-	+	+	-

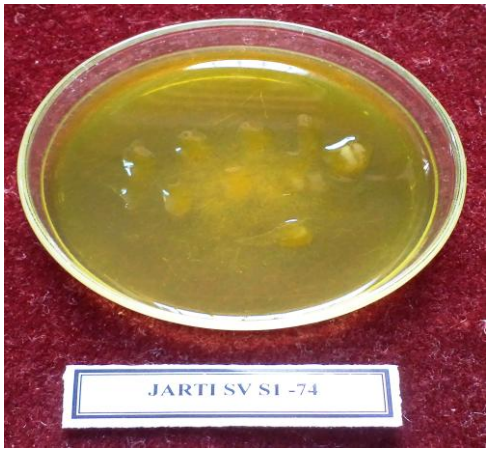
(-) = negative, (+) = positive

Biochemical study

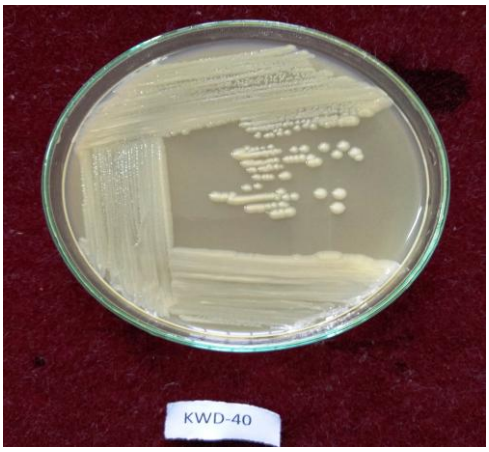
Top performing isolates were tested for their behavior towards different biochemical tests. All the tested isolates were found positive towards catalase and citrate utilization, negative towards indole production and gelatin lequification. However some isolates were found positive and some found negative towards urease production, nitrate reduction, acid production, MR-VP for acid production (Table 4).

Table 4: Selective biochemical test of *Gluconacetobacter strain* isolates.

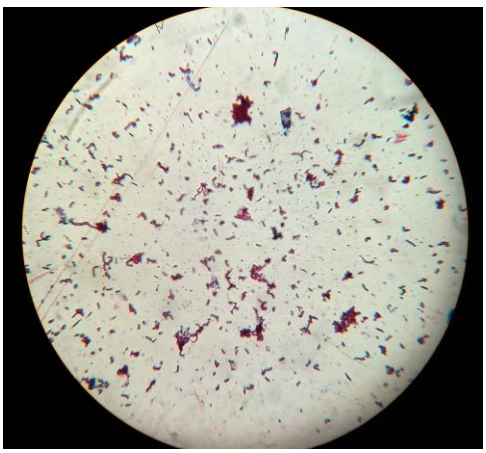
Isolates Code		Biochemical test								
		Gelatin test	Catalase	Urease	Nitrate reduction	Indole	Methyl red	Voges-Proskauer	Citrate Utilization	TSI test
1	JKWD-49	-	+	+	-	-	-	-	+	-
2	UKWD-40	-	+	+	-	-	+	-	+	-
3	JKWD-74	-	+	-	-	-	+	+	+	-
4	KKWD-16	-	+	+	+	-	-	-	+	A



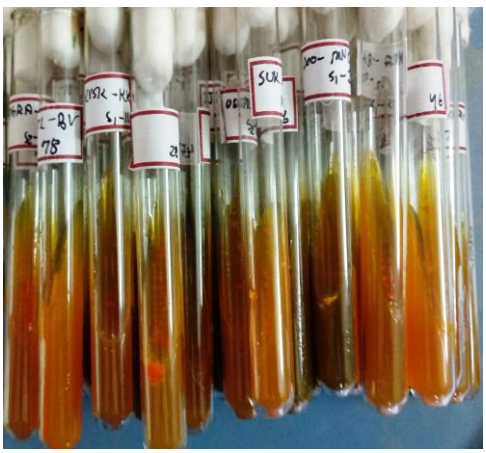
LGI Medium Growth *Gluconacetobacter*



GYC Medium Growth of *Gluconacetobacter*



Microscopic observation of *Gluconacetobacter*



LGI Slant Culture of *Gluconacetobacter*

Fig 1: Colony morphologies and microscopic observation of *Gluconacetobacter strain* isolates.

Discussion

Forty isolates of dizotrophic bacteria *Gluconacetobacter* were isolated from sugarcane stem and rhizosphere. Their atmospheric nitrogen fixing capacity was tested in N-free media. Top four N-fixers were further characterized. All the collected isolates have shown nitrogenase activity and were found gram negative, ellipsoidal or rod shaped. According to Bergey's Manual of Systematic Bacteriology (Volume-1), 2005 the characteristics of collected isolates are similar to that of *Gluconacetobacter*. Reis *et al*. (2004) [7] and Kadere *et al*. (2008) [3] also reported that the colonies of *Gluconacetobacter* bacterium are pale yellow in colour, convex and gram negative in nature.

At present the above effective isolates are being tested for their N-fixing capacity in farmers fields. The results are found very encouraging.

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