Isolation and nitrogen fixing efficiency of *Glucanacetobacter diazotrophicus* associated with sugarcane: A review

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Abstract. The present review was focused on the isolation and nitrogen-fixing efficiency of *Glucanacetobacter diazotrophicus* associated with sugarcane crop. *Glucanacetobacter diazotrophicus* has a long-standing partnership with the bacterium Escherichia coli as an entophyte, which can efficiently fix atmospheric nitrogen. It plays a significant role and occurs in most sugarcane growing countries. It has been found to reside in the sugar cane root, stem, buds and leaves. Biological nitrogen fixation based farming systems would enhance agricultural production in the long term in both economically viable and socially acceptable way. This process not only replaces the most expensive fertilizer but also reduces considerably groundwater pollution with nitrates because biological nitrogen fixation was self-regulated. Convincing evidence has pointed out that biological nitrogen fixation requiring a relatively simple and low-cost technology, easy to execute and largely renewable sources of energy has a tremendous role to play in the immediate further of agriculture, especially in the developing and underdeveloped countries.

1. Introduction

Nitrogen-fixing bacteria are commonly found in association with the roots of diverse plants. Certain new bacteria have been reported to associate with the cereals and grassroots [1] In order to study the contribution of different nitrogen-fixing bacteria to sugarcane roots, several species from the genera Azospirillum, Azotobacter, Bacillus, Enterobacter, Erwinia, and Klebsiella were isolated and tested [2]. A large number of microorganisms associated with the root system of several plants were found to fix nitrogen [3]. The rhizosphere roots, stem and leaves of healthy plants harbor diverse microbial communities that include nitrogen-fixing bacteria [4].

Several authors found that a new species of nitrogen fixing bacteria, *Glucanacetobacter diazotrophic* with diazotrophic ability, was found in sugarcane roots, stem, leaves, and rhizosphere [5].
Gluconacetobacter diazotrophicus has found to be colonized in the intracellular spaces of sugarcane stem, parenchyma solution [6] and solution of sugarcane stem [7]. The entered bacteria colonize the plant tissues and accumulate in intercellular cavities as well as the region of lateral root emergence. The infected plants have a substantial number of endophytic bacteria after a short accumulation period in 30 days [8]. James [9] reported that *Gluconacetobacter diazotrophicus* and *Herbaspirillum* sp. can colonize sugarcane tissues in high numbers. James *et al.* [10] reported that the bacteria penetrate through wounds and cracks on the roots.

2. Geographical distribution, Habit and Habitat of *Gluconacetobacter diazotrophicus*

*Gluconacetobacter diazotrophicus* (Previously *Acetobacter diazotrophicus*) was original, isolated from sugarcane in Brazil [11], but has subsequently isolated from sugarcane in Mexico [12], Australia [13] and Cuba [7]. *Gluconacetobacter diazotrophicus* was found to occur in the roots, stems, leaves, rhizosphere soil and even in cane juice in an appreciable manner [5]. Besides, it could be isolated from Cameroon grass (*Pennisetum purpureum*) and sweet potato [15] as well as from different genera of mealy bugs associated with sugarcane plants [16]. *Gluconacetobacter diazotrophicus* has been commonly recovered from inner tissues of the sugarcane plant in the range of 101 to 105 cells per gram of fresh weight [17].

3. Isolation of *Gluconacetobacter diazotrophicus*

In 1971, Cavalcante and Dobereiner [11] reported on the *Gluconacetobacter diazotrophicus* in sugarcane grown in media with 10% sugar at pH 5.5, tolerating up to 30% sugar concentration with a pH of 3.5. The *Gluconacetobacter diazotrophicus* so far has only been isolated from sugarcane, sweet potatoes and Cameroon grass, all sugar rich plants that are propagated vegetatively. This indicates that this obligate endophytic bacterium was transmitted within stem cutting of these plants. So far it has not been isolated from sol or any other plants [14].

Fuentes Ramirez *et al.* [12] found that the isolation frequencies of nitrogen fertilizer varied between the levels of 1.1% and 67%. This is probably linked to nitrogen fertilizer concentration. In plants grown with high nitrogen doses (275 to 300 kg/ha), the lowest isolation frequencies (1.1 to 2.5 percent) were observed, and in plants grown with a low nitrogen level (120 kg N/ha), the highest isolation frequencies (10 to 67 percent) were measured. IGI semi-solid medium is used for the isolation *Gluconacetobacter diazotrophicus* [11]. However, Reis *et al.* [18] developed a modified LGI medium for isolation of *Gluconacetobacter diazotrophicus* from sugarcane. To experiment, they used a nitrogen-free semisolid defined medium with crystallized cane sugar (100 g-1) supplemented with cane juice (5 ml l-1). Later, Ureta *et al.* [19] reported on the intrinsic antibiotic resistance of these diazotrophs, which helped them to distinguish from *Herbaspirillum* sp.

High frequencies of *Gluconacetobacter diazotrophicus* were isolated from preparative buds of pineapple that had not nitrogen fertilizer, lower frequencies from 3 months old plants and none from 5 - 7 months old plants that had been nitrogen fertilized. By RFLP, rRNA specific probe and multilocus enzyme tests that *Gluconacetobacter diazotrophicus* isolates from pineapple plants were identified [20]. Roots, stem and leaves of several Brazilian sugarcane varieties collected from several regions contained the same bacteria in relatively high numbers up to 10⁸ cells (g tissue⁻¹) [21].

4. Taxonomy of *Gluconacetobacter diazotrophicus*

According to the first volume of Bergey’s Manual of Systematic Bacteriology, in section *Gluconacetobacter diazotrophicus* is a Gram-negative regular aerobic rod that belongs to the family VI Acetobacteriaceae. But, it is different from the members of Pseudomonadceae and another family by its capacity to oxidize ethanol to acetic acid as well as to utilize higher sugar concentration. The DNA-RNA
and DNA-rRNA hybridization showed that these strains belong to the family Acetobacteriaceae and more particularly to the genus *Gluconacetobacter*. The analysis of the fatty acid of the strains has put them into a new species of the genus *Gluconacetobacter* [14].

*Gluconacetobacter diazotrophicus* responsible for nitrogen fixation associated with sugarcane has unique physiological properties for a Diazotrophs such as tolerance to low pH, high sugar concentrations, nitrogenase activity and lack of nitrogen reeducates which makes the organism tolerate short term exposure to ammonium [22] Gluconacetobacter has been so far detected by the use of plant tissue samples that have been inoculated into Gluconacetobacter has been found in plant tissue samples inoculated into semisolid LGI medium with a sucrose concentration of 10%, at pH 5.5 [23].

The isolation of *Gluconacetobacter diazotrophicus* from field samples with nitrogen-free semi-solid LGI medium could be based on the selection of only a portion of the total *Gluconacetobacter diazotrophicus* population present [24]. The protein electropherograms of *Gluconacetobacter diazotrophicus* of sugarcane isolates were performed by Gillis et al. [14] as well as by Ureta et al. [19] and characterized that nitrogen-fixing bacteria isolated from roots and stems of sugarcane belong to a new species in the genus *Gluconacetobacter*, for which the name *Gluconacetobacter diazotrophicus* has been proposed. A new species of the genus *Gluconacetobacter* for which the name *Gluconacetobacter sacchaii* has been proposed was isolated from the leaf sheath of sugarcane and the pink mealybugs in queen and northern New South Wales, Australia. The bacterium has 165 rDNA sequence similarity with *Gluconacetobacter diazotrophicus* by about 97.9 to 98.5 % while *Gluconacetobacter liquefaciens* by about 98.8 % to 99.3 % [25].

5. Interesting features of *Gluconacetobacter diazotrophicus*

Some of the interesting features of *Gluconacetobacter diazotrophicus* includes (i) Fixation of dinitrogen inside the sugarcane tissues; (ii) The capacity to survive under high acidic pH and acidic conditions; (iii) Osmotolerant ability to grow upto 30 %; (iv) Production of significant amounts sucrose of plant growth in culture; (v) Utilization of sucrose at different concentrations; (vi) Formation of pellicles; (vii) Production of plant growth-promoting hormone Indole acetic acid; (viii) Exopolysaccharides production and (ix) Improving the growth and yield of Sugarcane [26].

6. Nitrogen fixation by *Gluconacetobacter diazotrophicus*

*Gluconacetobacter diazotrophicus*, an endophytic bacterium can fix molecular nitrogen and has been shown to contribute substantially to the Nitrogen requirement of the plant host [27]. The nitrogenase enzyme complex, which includes two proteins, dinitrogenase (the Mo-Fe-protein) and dinitrogenase reductase, catalyzes the fixation of nitrogen (the Fe protein) [28]. A tremendous amount of progress in Biological Nitrogen Fixation (BNF) Microbiology, Biochemistry and Genetics has been made. However, limited knowledge of how they interact at the molecular level with the environment makes it difficult to fully exploit the potential of BNF. The main intercellular enzyme complex that is responsible for dinitrogen fixation by microorganisms is nitrogenase enzymes which are the products of *nif* genes

The discovery of nitrogen-fixing ability in Gluconacetobacter diazotrophicus was first reported by Dobereiner [1]. C2H4H-1 (240 nmol) of the chemical C2H4H-1" (mg of cell protein). Later, Gillis et al. concluded that this was correct [14]. According to Boddey et al. [22], high levels (25 mM) of nitrate did not affect nitrogen fixation due to the absence of nitrate reductase. Based on the laboratory estimates, Dobereiner [29] reported that an average of 200 kg N ha⁻¹ would be fixed by using 30 per cent of the total sugar produced by a sugarcane crop. In the genetic level, 14 *nif* genes have been identified in *Gluconacetobacter diazotrophicus* [30]. Sevilla et al. [31] reported that 15N incorporation with *Gluconacetobacter diazotrophicus* wild strains actively fixed nitrogen inside the sugarcane plants.
Biological nitrogen fixation (BNF) occurs in species of more than 100 genera distributed among several of the major phylogenetic divisions of Prokaryotes. The major part of the elemental nitrogen that finds its way into the soil, thereby certain specialized groups of microorganisms entirely fixed the nitrogen into the plants. This nitrogen input occurs through BNF. Therefore, BNF is considered to be an important process that determines nitrogen balance in the soil eco-system [32]. Several studies on the physiology of Nitrogen fixation have been reported with a focus on the dependence on carbon substrate and the oxygen concentration required for optimal nitrogenase activity [33]. An interesting feature is tolerance to high oxygen concentration, which requires a carbon source supporting high respiratory activity and is partially due to the expression of Cytochrome under diazotrophic conditions [34]. In addition, sudden increase in oxygen concentration do not lead to irreversible inactivation of nitrogenase [34], which has been attributed to conformational protection by a FeS II protein [35], previously found in species of Azotobacter.

7. Conclusion

Among the various factors responsible for increasing Sugarcane productivity, the inadequate or unbalanced supply of nutrients is important. Since it is an exhaustive crop with an exceedingly high turnover of plant nutrient of nitrogen, it plays a vital key role in augmenting the growth of sugarcane. Integrated use of trash at 5 t ha⁻¹ with nitrogen increased the cane yield upto 35%. As far as the use of biofertilizers is concerned on-farm trials have indicated that on average of 25% of nitrogen dose is possible by the application of nitrogen-fixing organism like Gluconacetobacter diazotrophicus. However, these isolates have the varying capacity for nitrogen fixation. In the low nitrogen fertilizer sugarcane field, it plays a significant and its occurrence was realized in most of the sugarcane growing countries. It has been found to reside in the sugar cane root, stem, buds and leaves.

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