"Development of bioformulation using beneficial plant-associated microorganisms for protection from fungal pathogens and yield improvement of *Stevia rebaudiana*"

SUMMARY OF

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Use of chemical based fertilizers and pesticides for enhanced the crop production are a major concern worldwide due to associated environmental pollution and health hazards. Hence, there is an urgent need to develop an eco-friendly solution to enhance crop production and promote sustainable agricultural development. The future is only for the eco-friendly measures which not only lead to sustainable and pollution free environment but also development of a market sound product instilling confidence amongst the end user. *B. safensis* is an eco-friendly tool for promoting plant growth and use as biofertilizer. P-WBF based *B. safensis* bioformulation showed pronounced increase in the growth parameters and metabolites/bioactive components of *S. rebaudiana* and proved to be an alternative option for chemical fertilizers. *B. safensis* STJP possessing a bouquet of PGP characters and applied as novel paneer whey based not only enhanced the growth but also the stevioside content in Stevia plants by providing an array of nutrients. The result of present study proposes that *B. safensis* with PGP properties could be a promising tool for enhancing the livelihood for farmers through organic farming of *S. rebaudiana*. The exploitation of such PGP strains as a cheap and effective bioformulation based on organic wastes could be a better option for sustainable agriculture simultaneously leading to production of healthy food, reducing health problems related with chemical fertilizers, and providing green metabolites to pharmaceutical industry.

Stevia rebaudiana is an ayurvedic herb, frequently known as sweet leaf or candy leaf or honey leaf or sweet herbs or Meethi Patti. It belongs to the Asteraceae family and grows up to 1-meter height. It is popular worldwide for its natural non-calorie high sweetener value. *S. rebaudiana* cultivation is common in certain regions of South

America, mainly in Brazil and Paraguay. Moreover, the plant has also a wide range of potential biomedical applicability viz. anti-inflammatory, anti-hyperglycaemic, anti-tumor, hepatoprotective activity, immunomodulation, blood sugar control, hypertension reduction and in treating skin disorders. *S. rebaudiana* also contains essential nutrients such as iron, silica, cobalt, manganese, calcium, magnesium, selenium, tin, zinc, vitamin C, beta-carotene, niacin, thiamine, and riboflavin. Beside essential elements, the extracted leaves of stevia contain caffeic acid, isoquercitrin, phenylpropanoids, quercetin, scopoletin, umbelliferone. In addition to high medicinal value, it is a natural sweetener with zero calories and low glycemic index. Its sweetener property depends on the alkaloid, diterpene glycosides (DGs). The DGs consist of several compounds like stevioside, steviolbioside, rebaudioside A, B, C, D, E and dulcoside, which have been extensively explored. Among them, stevioside is in limelight due to its harmless sweetener quality. Stevioside is slightly bitter in taste, nonfermentable, non-caloric and 300 times sweeter than normal/table sugar with a long shelf life. Leaf of *S. rebaudiana* contains the highest content of stevioside (6-18%) and gradually it decreases in other parts of the plants. The European Food Safety Authority (EFSA) along with the Food and Agriculture Organization (FAO) as well as the World Health Organization Expert Food Committee (WHO-EFC) have approved stevioside as safe for human use as an alternative to sugar especially in case of diabetes patients. The priceless medicinal properties of stevioside lead to an upturn in *S. rebaudiana* cultivation all over the world.

In the present study, rhizobacterial isolate STJP from the rhizosphere of *Stevia rebaudiana* was identified as a *Bacillus* safensis STJP on the basis of phenotypic, biochemical, and 16S rRNA gene sequencing. Afterward, isolate *Bacillus* sp. STJP produced a significant quantity of indole 3-acetic acid (30.59 µg/ml), gibberellic acid

(8.22 µg/ml), and siderophores production. Further, isolate STJP was able to solubilized potassium (4.41 µg/ml), zinc (ZnO: 5.40 µg/ml, $ZnSO_4$: 3.20 µg/ml, $ZnCO_3$: 3.80 µg/ml), and phosphate (610.33 µg/ml). On the basis of good phosphate solubilization ability, isolate was checked phosphatase activity and found to be 81.45 U/ml after 96 h.

Isolate STJP did not produce HCN production as confirmed by a change of Whatmann filter paper from yellow to red. Further, bacterium strain STJP was able to produce salicylic acid 12.80 µg/ml. Isolate STJP was tested for lytic enzymes viz. chitinase (75.58 nmol/min/mg), β-1, 3 glucanase (148.50 nmol/min/mg), and cellulase. These enzyme help in the breakdown of fungal cell wall.

Fungal phytopathogens resulted in the reduction of crops and using chemical fertilizers, and pesticides is not effective method by eco-friendly, sustainable and in the health by reducing of soil quality, enhancement of soil salinity and environmental pollution. Currently, public is demanding eco-friendly, sustainable tools for growth and productivity of crop from protection against fungal pathogens. In the current study, *B. safensis* STJP showed significantly antagonistic activity against *Alternaria alternata*. The growth reduction of *A. alternata* by *B. safensis* STJP has been confirmed by SEM. It did not show pathogenic activity, thus *B. safensis* STJP is a safe candidate as the development of biopesticide to fight the current challenge of food security.

B. safensis strain STJP produced a large number of volatile metabolites as confirmed by thin layer chromatography, FT-IR, and GC-MS basis. These metabolites contained a wide range of organic compounds viz. fatty acid, alcohol, phenol, and other aliphatic compounds. Among all, a compound 2, 4-bis (1, 1-dimethylethyl)/2, 4-di-tert-butylphenol showed highest growth inhibition rate of *A. alternata* at 40 ppm

concentration. The production of volatile concentration by *B. safensis* could be effective in controlling the fungal pathogen *A. alternata* in the soil and other related area where volatile metabolites will be reached.

The cocktail of PGP and biocontrol characters helped in the development of a suitable bioformulation for enhancing yield and stevioside content of *S. rebaudiana*. In the greenhouse experiment, treatment with STJP showed significant increase in the plant growth parameters viz. number of leaves, fresh and dry weight, plant length of *S. rebaudiana* as compared to control plants. Combine treatment of STJP and mycorrhiza showed highest plant growth parameters as compared to control plants. Chlorophyll (a and b), carbohydrate, flavonoid content, and antioxidant activity was further enhanced by the treated plants with STJP. In addition, this treatment was able to enhancement of nutrients uptake in *S. rebaudiana* and soil nutrients as compared to control and *A. alternata* treated plant. However, in the pot study, integrated approach of using STJP and along with mycorrhiza enhanced highest growth parameters, physiological parameters, nutrients uptake and stevioside content in the *S. rebaudiana* as compared to control. Isolate STJP and combine treatment of STJP+mycorrhiza were recoded maximum stevioside content in Stevia plant as compared to control and negative control (*A. alternata*). Stevioside is a key plant metabolite having wide applications in various industries. Enhancement of stevioside yields that too through eco-friendly biological approach can be a major breakthrough for stevioside production.

In the present study, bacterium STJP was taken as plant growth promoting microbe with multiple PGP characters for making bioformulation to enhance the growth of *S. rebaudiana*. Paneer-whey (a dairy waste) based bioformulation (P-WBF) was developed utilizing the isolate STJP on the basis of bacterial shelf-life, ease of availability, nutrient characteristics, and economical way and inspected for the quality and ability to enhance the growth, nutrients uptake, and stevioside content in *S. rebaudiana*. A combine P-WBF+mycorrhiza treatment in *S. rebaudiana* enhanced fresh and dry weight by (91.36%, 78.94%), (73.41%, 104.82%) and (89.02%, 184.22%) after 30, 60, and 90 days, in comparison with a control plant, respectively. Further, P-WBF + mycorrhiza treatment resulted in the maximum plant length and number of leaves enhancement (by (105.03%, 98.55%), (94.55%, 93.22%) and (130.34%, 81.22%), respectively), as compared to the control after 30, 60, and 90 DAS. Afterward, as in the case of parameters such as chlorophyll, carbohydrate, flavonoid and antioxidant activity, maximum enhancement was recorded when *S. rebaudiana* treated with P-WBF and mycorrhiza. The treatment with STJP+mycorrhiza showed significantly high stevioside content (120.62%) in *S. rebaudiana* as compared to control. Alone STJP treatment resulted in better stevioside quantity in Stevia plants when compared with other control (95.39%) and mycorrhizal (11.59%) treated plant. Overall results suggested that P-WBF+mycorrhiza is a reliable method for enhancement of number of leaves, fresh and dry weight, and plant height and stevioside content of *S. rebaudiana*.

P-WBF+mycorrhiza was applied on Stevia plants and resulted in 80.86% enhancement of P in the plant when compared with control. P-WBF and mycorrhiza was positively affected in the P content as compared to uninoculated plants. Zn and K nutrients uptaken in *S. rebaudiana* were significantly enhanced when applied with P-

WBF+mycorrhiza. The quantity of Zn and K was enhanced by 44.54% and 122.70% respectively, by P-WBF+mycorrhiza treated *S. rebaudiana* than that of without inoculated plants. Mycorrhiza and P-WBF alone treatments were recorded higher Zn (19.62%, 38.62%) and K content (56.11% and 95.63%) as compared to control. P-WBF+mycorrhiza+*A.alternata* was enhanced significantly in Zn (106.45%) and K (97.08%) concentration in the comparison with the negative control plant. Mycorrhiza+*A.alternata* treated plants showed 33.33% and 110.26% increment in the uptake of K and Zn when it was compared with negative control plants. Overall, P-WBF+mycorrhiza recorded a good method for uptake of nutrients (P, K and Zn) in *S. rebaudiana* from the soils.

After harvesting of *S. rebaudiana*, soil nutrients in the soil was checked and observed that K, P, Zn, C, and N of P-WBF+mycorrhiza treated soils was 100.89, 39.16, 366.66, 67.10, and 74.72% higher than without any treatment (control soils). However, P-WBF+Mycorrhiza inoculated in Stevia plants was increased K, P, Zn C, and N in the soils, the result was compared with negative control and without treated plant (Table). Overall, P-WBF+mycorrhiza was found to be a good method in the enhancement of soil nutrients (K, P, Zn C, and N).

Transcript accumulation profiling of candidates genes exhibited two groups, 1) upregulation 2) down-regulation in all the treatments. All genes were calculated to transcript levels with the reference gene of act and 18S rRNA. Among all three genes, UGT76G1 and UGT85C1 showed up-regulation expression in P-WBF treated plants and this result was confirmed more accumulation of SGs, whereas UGT74G1 was found to be down-regulation in P-WBF treated plants, means it was unable to enhance the accumulation of SGs in plants. UGT76G1 and UGT85C1 showed more than 4.3 fold and 2.4 fold changes in comparison with control, respectively. Overall, two genes

(UGT76G1 and UGT85C1) was involved in the up-regulation of expression and transcript accumulation. This novel approach can be a clean technology and also important for utilization of waste in preparation of bioformulation and enhancement of crop yield by an eco-friendly manner leading to sustainable agriculture. The exploitation of *B. safensis* having PGP character and biocontrol activity as a cheap and effective bioformulation for sustainable agriculture simultaneously leading to the production of healthy food, reducing health problems related with chemical pesticides, and providing green metabolites to pharmaceutical industry.