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Influence of Yeast Bioinoculant Isolated from Indian Date Palm Tree (*Phoenix sylvestris*) Sap on the Health of Wheat Crop and Soil

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ABSTRACT

In this study, three promising yeast isolates were isolated from the sap of the Indian date palm tree (*Phoenix sylvestris*) and characterized by biochemical tests and 18S rRNA gene sequencing. They were confirmed as *Saccharomyces cerevisiae* and were designated as strains PYS-1, PYS-2, and PYS-3. These confirmed strains were used for the preparation of bioinoculants. Bioinoculant was prepared and applied to wheat crops, and the effect of Bioinoculant. Statistical analysis is carried out using analysis of variance (ANOVA), and it is found that the absorbance of chlorophyll, protein, and Indole Acetic Acid (IAA) content is significantly increased. The treatment of bioinoculant showed that crops significantly increased chlorophyll, protein, and IAA content. Further, we applied bioinoculant on the soil and measured the soil's humus content before and after the treatment of bioinoculant. Then, a paired t-test was applied to check the effectiveness of the treatment, and it was found to significantly increase humus content in the soil. The use of bioinoculants is an economically feasible and eco-friendly method.

INTRODUCTION

Since the inception of mankind, human beings have been using traditional plants to manage several ailments. One traditional plant, *Phoenix sylvestris*, is widely known as wild date palm. *Phoenix sylvestris* is commonly known as Indian date and is native to India and Southern portions of Pakistan. *P. sylvestris* is widely distributed in India, Pakistan, Myanmar, Nepal, Bhutan, Bangladesh, Mauritius, China, and Sri Lanka. Currently, the pest is reported in c. 15% of the coconut-growing countries and nearly 50% of the date palm-growing countries (Faleiro 2006). It is mostly found in Rajasthan, Gujarat, Himachal Pradesh, and Haryana states in India. It is traditionally important and known for its nutritional values worldwide (Barh & Mazumdar 2008). It is a rich source of carbohydrates, phenols, amino acids, tannins, flavonoids, alkaloids, terpenoids, dietary fibers, essential vitamins, and minerals. Sap is a Phloem, or sieve-tube sap, which is the fluid carrying sugar from roots to other parts of plants. Sap is a watery fluid of plants. Most tapped palm trees give a sap rich in sugar (10-20%). The farmers achieve the palm mainly for sap production, with which sugar-based secondary goods are manufactured. The sap is used fresh as a drink or after some processing as molasses and/or alcoholic beverages (Francisco & Scott 2013). Seven diversified sites support the palm as its territory; most palms (20.40%) occur in orchards (Chowdhury et al. 2008). Sap can be used for fermentation as it contains sugar. Sweet sap is consumed fresh, processed into syrup or sugar, or fermented into alcohol or vinegar. Yeasts are eukaryotic, single-celled microorganisms classified as members of the fungus kingdom. Yeast sizes vary greatly, depending on species and environment, typically measuring 3–4 µm in diameter, although some yeast can grow to 40 µm in size. The yeast species *Saccharomyces cerevisiae* converts carbohydrates to carbon dioxide and alcohol through fermentation. Microbial inoculants, or bioinoculants, are agricultural alterations that use beneficial rhizospheric or endophytic microbes to promote plant health. Bioinoculant may consist of either a single or consortium of mixed microbial populations, which are substances containing live microorganisms, which, when applied to plant surfaces, seeds, roots, or soil, colonize the rhizosphere or the interior of plants and help to improve soil fertility while also stimulating plant growth by increasing the availability of plant nutrients and growth substances to the host crops (Suyal et al. 2016, Vessey 2003) like *Triticum aestivum*. Wheat (*Triticum aestivum*) is a crop widely cultivated for its seed, a cereal grain that is a worldwide

staple food. The many species of wheat together make up the genus *Triticum*; the most widely grown is common wheat (*T. aestivum*). Wheat is an important source of carbohydrates. Indole acetic acid (IAA) is one of the greatest physiologically active auxins. IAA is a common product of L- L-tryptophan metabolism produced by several microorganisms, including Promoting Rhizobacteria (PGPR). The plant growth parameters were found to be enhanced by the mixed inoculation of two groups of R and E bacteria compared to individual inoculations (respectively 33.7 and 37.8% increase in root and shoot dry weight), suggesting that PGP rhizobacteria acted synergistically with PGP endophytes in phosphate solubilization (Emami et al. 2019). IAA helps produce longer roots with more root hairs and root laterals involved in nutrient uptake. Soil fertility refers to the capability of soil to sustain agricultural plant growth, *i.e.,* to provide plant habitat and result in sustained and consistent yields of high quality. The organic matter (OM) content influences many soil properties, such as the capacity of soil to supply N, P, S, and trace elements; infiltration and retention of water; degradation of aggregation; and overall soil structure. Cation exchange capacity, soil color.

MATERIALS AND METHODS

Collection of sap sample: The sap was extracted and collected by tapping. The sap sample was collected from Bhor, Pune.

Isolation of yeast isolates from sap: Sap was collected, and 2-5mL of the sample was transferred into Sabouraud's dextrose broth (SD) and Yeast extract broth (YE) for the enrichment and incubated at 30°C for 48h on a rotatory shaker. YE agar plates and SD agar plates were inoculated with enriched broth (Anyanwu et al. 2020). Then, plates were incubated at 30°C for 48 hours. The yeast nature of isolates was confirmed by microscopy.

Microscopic examination of isolates: Isolates were observed microscopically. The yeast was characterized for colony characters and gram nature.

Biochemical characterization of yeast isolates: The biochemical tests viz. sugar fermentation and utilization, oxidase, catalase, nitrate reduction, and IMViC tests were used for the characterization of promising isolates (Bergey & Holt 1994).

Preparation of bioinoculant: Promising isolates (10^8CFU/mL of each) were inoculated in sterile Yeast extract broth (100 mL). Enriched broth was kept on a rotatory shaker at 30°C for 48h incubation. Dilutions were prepared from incubated broth, spread on YE plates, incubated at 300°C for 48h, and colonies were counted of each isolate inoculated to study their compatibility.

Growth curve: Yeast growth is measured in a shaken conical flask by determining the optical density (OD) at 600 nm. Then, a growth curve is built by plotting OD versus time. Each of the four flasks of yeast extract broth of 100 mL, one as control. The three different isolates were then inoculated in each broth. The OD of each flask was recorded hourly from zero hour onwards. The graph was then plotted as a growth curve.

Estimation of photosynthetic pigment (Chlorophyll) content: The chlorophyll (a and b) content of the mature leaves of the test plant *Triticum aestivum* was estimated by the method of (Liang et al. 2017, Sadasivam & Manickam 1996)

Estimation of protein of test plant: Estimation of protein content was done by Lowry's method (Lowry et al. 1951).

Quantification of Indole Acetic Acid (IAA) of isolates by spectrophotometric method: It was quantified by Salkowski's method (Emami et al. 2019).

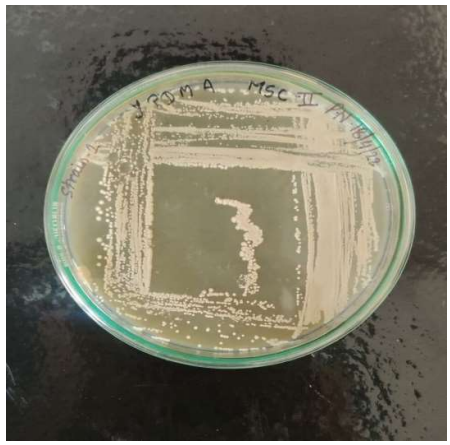
Estimation of the soil fertility- humus content of soil: It was determined by rapid dichromate oxidation technique (Walkley & Black 1934).

18S rRNA gene sequencing: The promising yeast isolates were identified presumptively per Bergey's Manual of Determinative Bacteriology based on physical, biochemical, and microbiological characteristics. All were further identified based on 18S rRNA gene sequencing using universal primers. Pure cultures were given to NCMR-Pune, India, and NCBI server (http://www.ncbi.nlm.nih.gov/BLAST) to check the similarity.

RESULTS AND DISCUSSION

Isolation of yeast: Yeast was isolated on Sabouraud's dextrose agar and yeast extract agar. Three promising yeast isolates were selected for further studies (Fig. 1).

Biochemical characterization of Yeast isolates: Three promising isolates were biochemically characterized with reference to (Bergey & Holt 1994) using eleven biochemical tests (sugars, IMViC, Amylase, Catalase, and Oxidase). From Table 1, it is observed that two (PYS-1 and PYS-2) isolates showed positive for Sugars, MR, Catalase, and Oxidase and negative for Indole, VP Citrate and Amylase whereas One (PYS-3) isolate showed positive for Sugars like Glucose, Sucrose, Fructose and negative for maltose, MR, Catalase, and Oxidase positive and negative for Indole, VP, Citrate and Amylase. These isolates were identified as *Yeast* spp. at the genus level.



Photoplate 1: PYS – 1

Photoplate 2: PYS - 2

Photoplate 3: PYS - 3

Fig. 1: Growth of yeast isolates on Sabouraud's dextrose agar at 30°C for 48 h incubation.

Table 1: Biochemical characterization of selected isolates.

Sr. No.	Biochemical tests	Isolates		
		PYS-1	PYS-2	PYS-3
1.	Glucose	+	+	+
2.	Sucrose	+	+	+
3.	Fructose	+	+	+
4.	Maltose	+	+	-
5.	Indole	-	-	-
6.	MR	+	+	+
7.	VP	-	-	-
8.	Citrate	-	-	-
9.	Amylase	-	-	-
10.	Catalase	+	+	+
11.	Oxidase	+	+	+

+ Positive test and – Negative test

18S rRNA gene Sequence: The 18S rRNA sequences of all three promising isolates confirm that isolates were *Saccharomyces cerevisiae* strains.

***Saccharomyces Cerevisiae* Strain PYS-1**

AGAGATGGAGAGTCCAGCCGGGCCTGCGCT-
TAAGTGCGCGGTCTTGCTAGGCTTGTAAGT-
TTCTTTCTTGCTATTCCAAACGGTGAGAGAT-
TTCTGTGCTTTTGTTATAGGACAATTAAAACCGT-
TTCAATACAACACACTGTGGAGTTTTCATATCT-
TTGCAACTTTTTCTTTGGGCATTCGAGCAATCGG-
GGCCCAGAGGTAACAAACACAAACAATTTTAT-
TTATTCATTAAATTTTTGTCAAAAACAAGAAT-
TTTCGTAACTGGAAATTTTAAAATATTAAAAACT-
TTCAACAACGGATCTCTTGGTTCTCGCATCGAT-
GAAGAACGCAGCGAAATGCGATACGTAATGT-
GAATTGCAGAATTCCGTGAATCATCGAATCTTT-

GAACGCACATTGCGCCCCTTGGTATTCCAGGG-
GGCATGCCTGTTTGAGCGTCATTTCCTTCTCAAA-
CATTCTGTTTGGTAGTGAGTGATACTCTTTGGAGT-
TAACTTGAAATTGCTGGCCTTTTCATTGGATGT-
TTTTT

***Saccharomyces Cerevisiae* Strain PYS-2**

GGCAAGAGCATGAGAGCTTTTACTGGGCAAGAA-
GACAAGAGATGGAGAGTCCAGCCGGGCCTG-
CGCTTAAGTGCGCGGTCTTGCTAGGCTTGTAA-
GTTTCTTTCTTGCTATTCCAAACGGTGAGAGAT-
TTCTGTGCTTTTGTTATAGGACAATTAAAAC-
CGTTTCAATACAACACACTGTGGAGTTTTCAT-
ATCTTTGCAACTTTTTCTTTGGGCATTCGAG-
CAATCGGGGCCCAGAGGTAACAAACACAAA-
CAATTTTATTTATTCATTAAATTTTTGTCAAAAA-
CAAGAATTTTCGTAACTGGAAATTTTAAAATAT-
TAAAAACTTTCAACAACGGATCTCTTGGTTCTCG-
CATCGATGAAAAACGCAGCGAAATGCGATA-
CATATTTTTTTTTTTGCAGAATTCCGAGAAT-
CATCGAATCTTTGAATA

***Saccharomyces Cerevisiae* Strain PYS-3**

CTTTTACTGGGCAAGAAGACAAGAGATGGA-
GAGTCCAGCCGGGCCTGCGCTTAAGTGCGCG-
GTCTTGCTAGGCTTGTAAGTTTCTTTCTTGCTAT-
TCCAAACGGTGAGAGATTTCTGTGCTTTTGT-
TATAGGACAATTAAAACCGTTTCAATACAA-
CACACTGTGGAGTTTTCATATCTTTGCAACT-
TTTTCTTTGGGCATTCGAGCAATCGGGGCCCA-
GAGGTAACAAACACAAACAATTTTATTTAT-
TCATTAAATTTTTGTCAAAAACAAGAATTTTCG-
TAACTGGAAATTTTAAAATATTAAAAACT-
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GAATTGCAGAATTCCGTGAATCATCGAATCTTT-

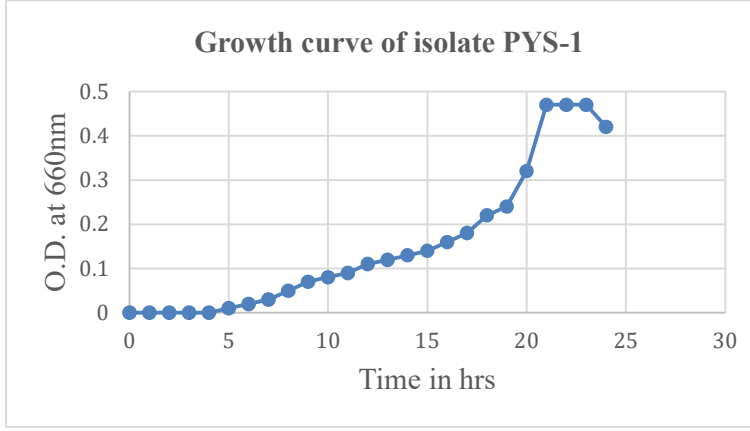

Fig. 2: Growth curve of promising isolate PYS -1.

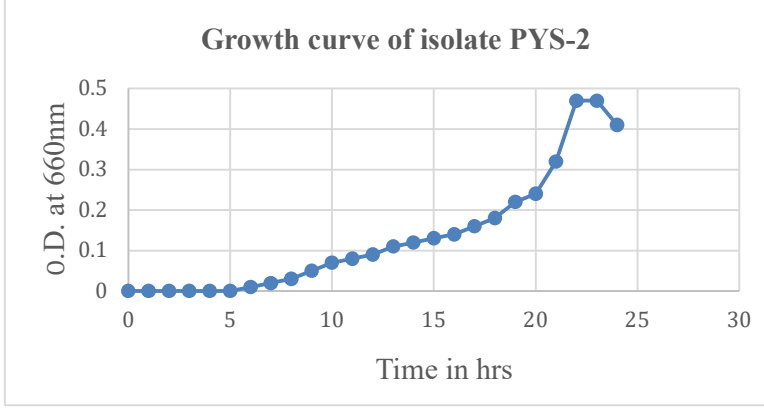

Fig. 3: Growth curve of promising isolate PYS -2.

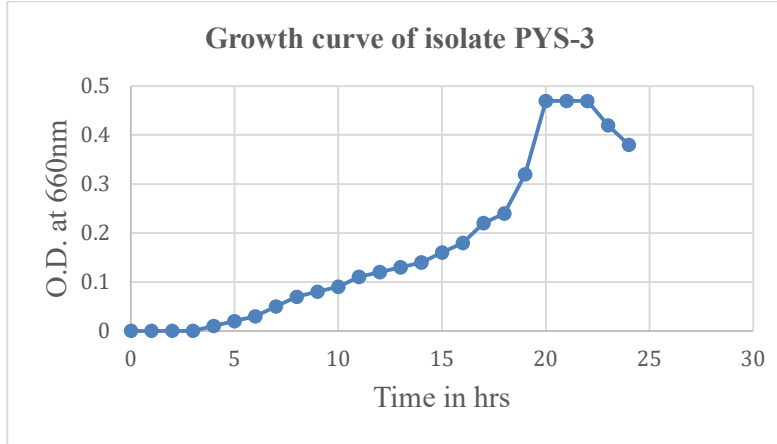

Fig. 4: Growth curve of promising isolate PYS-3.

GAACGCACATTGCGCCCCTTGGTATTCCAGGG-GGCATGCCTGTTTGAGCGTCATTTCCTTCTCAAA-CATTCTGTTTGGTAGTGAGTGATACTCTTTGGAGT-TAACTTGAAATTGCTGGCCTTTTCATTGGATGT-TTTTTTTTCCAAAGAGAGGTTTCTCTGCGTGCTT-GAGGTATAATGCAAGTACGGTCGTTTTAGGT-TTTACCAACTGCGGCTAATCTTTTTTATACTGAG-CGTATTGGAACGTTATCGATAG

Growth curve of promising isolates (PYS-1, PYS-2, and PYS-3): The growth of yeast isolates is observed in shaken conical flasks by determining the optical density (OD) at 660 nm. Then, a growth curve is a sigmoid graph built by plotting OD versus time, which allows identification and selection of the exponential phase and is fitted with the exponential growth equation to obtain kinetic parameters. Low specific growth rates with higher doubling times generally represent respiratory growth. Conversely, higher specific growth rates with lower doubling times indicate fermentative growth. In the growth curve of three (PYS-1, PYS-2, and PYS-3) promising isolates, found that up to 8, 5, and 4h time for PYS-1, PYS-1, and PYS-3 isolates, respectively, were in the log phase, indicating further ranged in between 4 to 8 h of incubation and there was no drastic difference in their growth pattern and could be compatible with each other in co-cultivation (Figs. 2, 3 and 4).

Bioinoculant (PYS-1, PYS-2, and PYS-3) was formulated as mentioned in Table 2. The bioinoculant was prepared in higher volume (in liters) for further studies (Fig. 5). This Bioinoculant was used as a biofertilizer, resulting in significant plant growth and soil fertility.

Effect of Bioinoculant on the growth of wheat *Triticum aestivum* and soil fertility: Bioinoculant was prepared from all three promising isolates (PYS 1, PYS 2, and PYS 3) and given to the crops and soil. The effect of bioinoculant was studied on the wheat crop (*Triticum aestivum*) compared to control. A positive effect on the crop is seen, as there is an increase in shoot length and root length for all three isolates and a mixture of them. Among the single treatments, the best upsurge for most growth parameters

Preparation of Bioinoculant of promising isolates

Fig. 5: Bioinoculant.

Table 2: Formulation of Liquid Bioinoculant.

Sr. No.	Isolates	No. of CFU.mL^{-1}
1.	PYS-1	10^8
2.	PYS-2	10^8
3.	PYS-3	10^8

Fig. 6: Effect of bioinoculant on crop (*T. aestivum)*.

(chlorophyll, protein, and IAA contents) was shown by *Triticum aestivum* plants compared to the control crop (Fig. 6).

Estimation of photosynthetic pigment (Chlorophyll) of *Triticum aestivum*: The total chlorophyll content of the wheat crop was measured by taking absorbance at 620nm

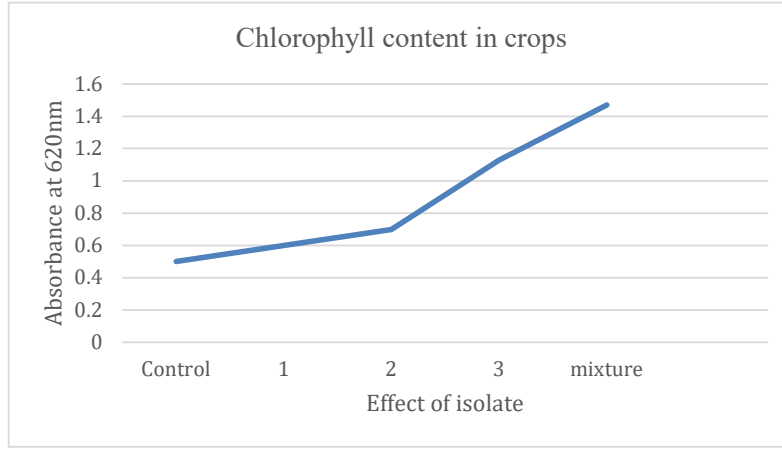

Fig. 7: Chlorophyll content at 620nm.

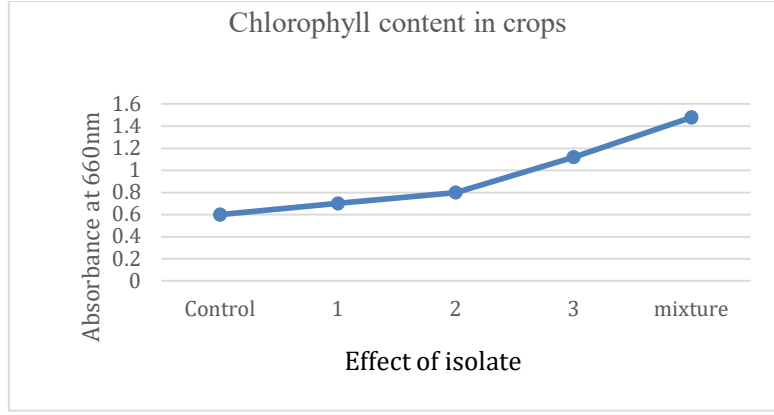

Fig. 8: Chlorophyll content at 660nm.

Table 3: Tukey's honest significant difference (HSD) test statistic for different pairs of isolates.

Pairs of isolates		q value at 620 nm	q value at 660 nm
Control	YS-1	1.326473065	1.543646179
Control	YS-2	4.863734571	5.196942137
Control	YS-3	8.224133002	7.975505259
Control	Mixture	13.0436518	13.5326315

and 660nm using a calorimeter. Results are plotted on the graphs as shown in Fig. 7 and Fig. 8, respectively.

The increasing chlorophyll in the inoculated crops probably resulted in higher photosynthetic rates and thus improved plant biomass. It has been shown in the study that total chlorophyll is more than that was reported earlier. The increase of chlorophyll that could lead to higher rates of photosynthesis is dependent.

Further analysis of variance (ANOVA) (Scheffe 1999) is carried out to test whether there is a significant difference in mean absorbance of chlorophyll at 620 nm and at 660 nm between the control isolates YS-1, YS-2, YS-3, and a mixture of these three isolates at 5% level of significance (l. o. s.) (α), *i.e.* $\alpha = 0.05$. The p-values are 0.0032 and 0.0030 for 620nm and 660 nm, respectively, which are less than α. So, there is sufficient evidence to conclude that the absorbance of chlorophyll is significantly different at 620 nm and 660 nm.

Hence, checking which isolate is more significant (effective) than others is necessary. To fulfill this requirement, we carried out Tukey's honest significant difference (HSD) test (Tukey 1949) and checked the significance difference between the control and other isolates. The values of test statistic q of Tukey's HSD test for control and different isolates are reported in Table 3, where the test statistic q is

$$q = \frac{\text{Abs(mean of control group} - \text{mean of isolate)}}{\sqrt{\frac{1}{2}} \times \text{SE of residual} \times \left(\frac{1}{n_1} + \frac{1}{n_2}\right)}$$

From the values of test statistic q, q value is highest for the pair Control and mixture. Therefore, a mixture of isolates is most effective, followed by isolates YS-3 and YS-2 at both 620 nm and 660 nm with respect to the extraction of chlorophyll.

Estimation of protein in *Triticum aestivum*: The protein content of enzyme extract is usually determined by Lowry's Method (Lowry et al. 1951) as amino acids give the exact quantification- the method developed by Lowry Reading was taken at 660nm, and a graph is plotted using the standard protein graph as shown in Fig. 9.

The results show that plant protein content significantly increased with bioinoculant injection compared to the control. Total protein content was determined according to the Lowry protein assay (Lowry et al. 1951), with reduced Folin reagent and subsequent colorimetric determination at 660nm.

Further analysis of variance (ANOVA) (Scheffe 1999) is carried out to test whether there is a significant difference in mean protein absorbance between Control isolates YS-1, YS-2, YS-3, and a mixture of these three isolates at 5% l. o. s. (α), *i.e.*, $\alpha = 0.05$. The p-values are found to be 0.02171, which is less than α. So, there is sufficient evidence to conclude that protein absorbance is significantly different.

Hence, to check which isolate is more significant (effective) than others. The values of test statistic q of Tukey's HSD test (Tukey 1949) for control and different isolates are reported in Table 4.

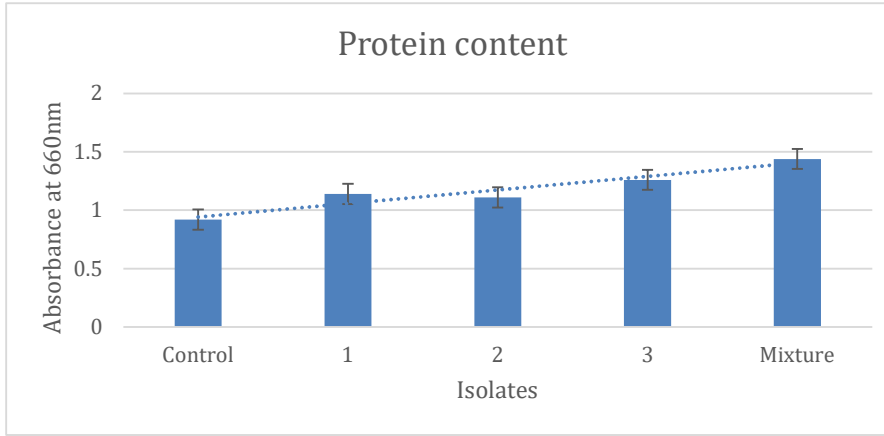

Fig. 9: Protein analysis chart.

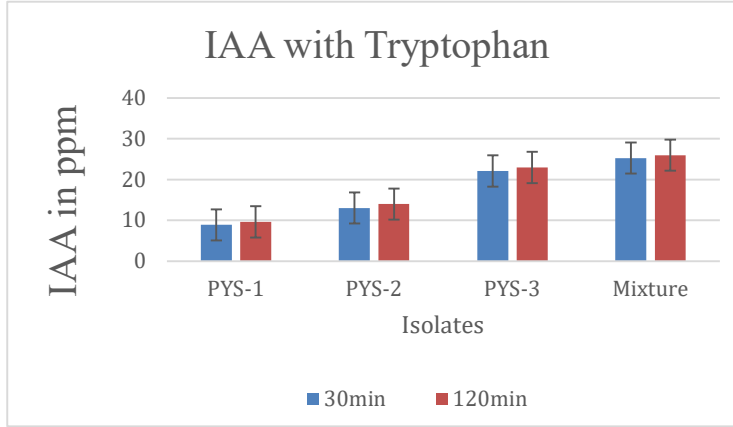

Fig. 10: IAA with Tryptophan analysis chart.

From the values of test statistics q, q value is highest for the pair Control and mixture. Therefore, a mixture of isolates is most effective, followed by isolates YS-3 and YS-2 with respect to the extraction of protein.

Estimation of Indole Acetic Acid (IAA) on *Triticum aestivum:* The significance of the study could be stated as the potential of these IAA-producing isolates will flourish the growth and ultimately IAA production in the field and prevent environmental pollution by avoiding excessive applications of chemical fertilizers. The IAA analysis chart is shown in Fig. 10.

All three isolates and their mixture were screened out for their productivity of IAA on a spectrophotometer. They all showed the ability to produce IAA with Tryptophan supplemented in the medium at 30 minutes and 120 minutes. These Endophytic bacteria are also helpful in developing liquid bioinoculants for economically convenient and sustainable agriculture. (Emami et al. 2019).

Further analysis of variance (ANOVA) (Scheffy 1999) is carried out to test whether there is a significant difference in mean absorbance of IAA at 30 minutes and 120 minutes between Control isolates YS-1, YS-2, YS-3, and a mixture of these three isolates at a 5% level of significance (l. o. s.) (α), *i.e.* $\alpha = 0.05$. The p-values are found to be 6.98×10^{-11} and 6.62×10^{-10} for 30 and 120 min, respectively, which are less than α. So, there is sufficient evidence to conclude that the absorbance of IAA is significantly different at 30 minutes and 120 min.

Hence, checking which isolate is more significant (effective) than others is necessary. We, therefore, carried out an HSD test and checked the significance difference between controls and other isolates. The values of test statistic q of Tukey's HSD test (Tukey 1949) for control and different isolates are reported in Table 5.

From the values of test statistics q, q value is highest for the pair Control and mixture. Therefore, a mixture of isolates is most effective, followed by isolates YS-3 and YS-2 at both 30 minutes and 120 minutes with respect to extraction of IAA.

Effect of bioinoculant on soil (Organic matter): The humus content of soil before and after applying bioinoculant (treatment) is reported in Table 6.

Table 4: Tukey's honest significant difference (HSD) test statistic for different pairs of isolates.

Pairs of isolates		q value
Control	YS-1	4.914942619
Control	YS-2	4.839328117
Control	YS-3	7.107763172
Control	Mixture	10.20795775

Table 5: Tukey's honest significant difference (HSD) test statistic for different pairs of isolates.

Pairs of isolates		q value at 30 minutes	q value at 120 minutes
Control	YS-1	3.841463511	1.140572822
Control	YS-2	14.35357865	12.24590758
Control	YS-3	36.76841271	28.30056821
Control	Mixture	43.49648101	34.17095266

Table 6: Estimation of organic matter.

Observation No.	Organic matter in $mg.mL^{-1}$ (Before treatment)	Organic matter in $mg.mL^{-1}$ (After treatment)
1	4.9	15
2	5.5	15.7
3	6.0	16.25

To check whether the treatment is effective or not, statistically, we carry out the paired t-test, and the corresponding p-value is found to be 0.000778, which is less than l.o.s. 0.05. Hence, it is concluded that the humus content of soil is increased in the soil after treatment of bioinoculant in comparison with control soil.

CONCLUSIONS

Three dominant yeast strains were screened out from the sap of wild date palm tree (*Phoenix sylvestris*) for preparation of bio-inoculant. All the isolates were characterized with their morphological characterization and genetically confirmed strains as *Saccharomyces cerevisiae* Strain PYS-1, *Saccharomyces cerevisiae* Strain PYS-2, *Saccharomyces cerevisiae* Strain PYS-3. Based on observed data, statistical analysis is carried out using ANOVA and paired t-tests. The results are reported in the respective sections. Regarding biochemical analysis like photosynthetic pigment, protein content, and hormone (IAA), it was clear that crop health (Triticum aestivum) has been increased with consortia. Also, concluding that there was an increase in the organic content of soil proving/ stating that this bio-inoculant is preferable. Microbial inoculants can minimize the negative impact of chemical input and consequently increase the quantity and quality of farm produce.

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