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ORIGINAL RESEARCH ARTICLE

Ammonium Accumulation in Culture Broth of Soil Yeast Isolates and Their Effects on Sorghum Plant

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ABSTRACT

A total of thirty yeast strains were isolated from various soil sources in PYG medium. The isolates were screened for the activity of nitrogen fixation on nitrogen fixing yeast medium. Among all yeast isolates, N3, N18, N21 and N24 could grow well in this medium and so they were selected for further study. According to their morphological characteristics, sugar assimilation and fermentation patterns and some biochemical characteristics, the selected yeast isolates could not be identified exactly. N3, N18 and N24 accumulated highest amount of ammonium concentration in raffinsoe containing media by giving 9.365 ppm, 4.774 ppm and 5.222 ppm of ammonium concentration. But, the highest amount of ammonium concentration accumulated by N21 was 2.263 ppm in the mannitol containing medium. In nitrogen free mineral broth medium, accumulated ammonium concentrations of selected yeast isolates were increased. N3, N18 and N24 accumulated 11.866ppm, 5.521 ppm and 8.027 ppm of ammonium concentration respectively in raffinose and N21 accumulated 5.089ppm of ammonium concentration in mannitol. On detection of accumulated ammonium concentration by Viscolor Alpha Ammonium Detection Kit, it was also found that they gave color development. In a pot trial study, maximum total nitrogen content in sorghum plant was recorded from inoculated treatment of N3 (1.52%) followed by inoculated treatment of N24 (1.51%).

Keywords: Nitrogen Fixation, Yeast, Ammonium Concentration, Total Nitrogen Content.

INTRODUCTION

Nitrogen, one of the most important nutrients, is a part of all living cells and is a necessary part of all proteins, enzymes and metabolic processes involved in the synthesis and transfer of energy. It is the nutrient that is most commonly deficient, contributing to reduce agricultural yields throughout the world. Molecular nitrogen or dinitrogen (N2) makes up four-fifths of the atmosphere, but is metabolically unavailable directly to higher plants or animals. It is available to some microorganisms through biological nitrogen fixation (BNF) in which atmospheric

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nitrogen is converted to ammonia by the enzyme nitrogenase.[1]

Nitrogen is needed by plants for the production of proteins, nucleic acids (DNA and RNA), and chlorophyll. Symptoms of N deficiency are general chlorosis of lower leaves (light green to yellow), stunted and slow growth, and necrosis of older leaves in severe cases. N deficient plants will mature earlier and crop quality and yield is often reduced. [2]

One of the most important factors for high yield of crops is the availability of nitrogen, phosphate and potassium.[3] Therefore, mineral fertilizers are used in order to increase crop yields. However, Extensive use of chemical nitrogen fertilizers may also inhibit the activity of natural nitrogen fixing microorganisms, thereby decreasing the natural fertility of soils.[4] In search for a solution to the problem, biofertilizer agents are of global interest.

Microbial fertilizer is able to increase yield and quality of crops without a large investment of money and labour.[5] Thus the extensive use of bio fertilizers would provide economic benefits to farmers improve the socioeconomic condition of the people and preserve natural resources.[6] Biofertilizer contain useful microorganisms that capable to supply a part of plant nutritional needs, and improve plant growth directly and indirectly. These bio-fertilizers enhance plant tolerance to environmental stresses such as water shortage and salinity.[7] Many attempts were made to prepare a bio-fertilizer from wastes using effective microorganism including bacteria and yeasts. Yeasts synthesize antimicrobial and other useful substances required for plant growth from amino acids and sugars secreted by bacteria, organic matter and plant roots.[8]

Yeasts are groups of unicellular fungi that belong to the phylum Dikaryomycota. The generation time is very short (about 90 min), so large populations of individuals can rapidly be grown and analyzed.[9] Yeast cell may multiply vegetative reproduction by budding (e.g. Saccharomyces) or fusion (e.g. Schizosaccharomyces) and sexual reproduction by Ascospore formation and basidiospore formation.[10, 11] Most of yeasts are useful organisms because of their fermentative ability, amino acid production, enzyme production, etc. They can also be used as many industrial products such as flavor enhancer, food additives, aspartame, sweetener, pharmaceutical, pesticide, etc. Nowadays, yeasts are widely used in biofertilizer preparation.[12]

Among the microbes, yeasts play two important roles in bio fertilizer production. The yeasts play a favourable effect upon the bacteria due to the change in pH value in the medium and the secretion of biologically active substances such as vitamins, enzymes, amino acid, etc. The second fact is that yeasts synthesize antimicrobial and useful substances for plant growth. Bioactive substances

such as hormones and enzymes produced by yeasts promote active cell and root division.[13] A growing number of studies indicate that plant root growth may be directly or indirectly enhanced by yeasts in the rhizosphere.[14, 15]

Saccharomyces cerevisiae is considered as a new promising plant growth promoting yeast for different crops. Recently, it became a positive alternative to chemical fertilizers safely used for human, animal and environment.[16] A wide diversity of soil yeasts have been researched for their potential as bio-fertilizers.[17, 18] Representatives of Candida, Geotrichum, Rhodotorula, Saccharomyces, and Williopsis are able to nitrify ammonium to nitrate via nitrite in vitro.[19] Whereas the ascomycetous genera Williopsis and Saccharomyces were able to oxidize elemental sulfur in vitro to produce phosphate, tetrathionate, and sulfate.[20] So, the use of yeast as a bio-fertilizer in agriculture has received considerable attention because of their bioactivity and safety for human and the environment.[21]

Therefore, this research was studied to isolate nitrogen fixing yeasts from soil sources and study on their ammonium accumulation for yeast based biofertilizer preparation.

MATERIAL AND METHODS

Sample Collection and Isolation of Yeasts

Soil samples were collected from Yangon Region, Kyaukse Township and Patheingyi Township of Mandalay Region in Myanmar.

To isolate yeast strains, one gram of collecting soil was mixed with 10ml of sterilized 0.9% NaCl solution in sterilized test tube and the mixture was kept standing for 1hour. 1ml of upper clear portion of the soil suspension was spread on PYG medium (peptone 2%, yeast extract 1% and glucose 2%) containing 0.05% chloramphenicol and the culture media were incubated at 37˚C for 24 hours.

Screening and Selection of Nitrogen Fixing Yeast Isolates

For the selection of nitrogen fixing yeast isolates, all yeast isolates from PYG media were cultured in nitrogen fixing yeast medium (0.2g K2HPO4, 0.2g MgSO4, 0.02g CaCl2, 15.0g Dextrose, 3 drops of 10% solution of FeCl3/l) and the culture plates were incubated at 37˚C for 5 days. After incubation, growth of yeast isolates on this media was observed. Yeast strains that could grow well in this medium were selected for further study.

Identification of Nitrogen Fixing Yeast Isolates

The colonies on PYG medium, nitrogen fixing yeast medium and nutrient medium were observed for colonial morphology. Cellular morphology of selected yeast isolates from all three media were examined by the Gram straining method under

microscope using a high power objective lens (1000x).

To determine the aerobic assimilation of carbon sources, the synthetic media was used.[22] After sterilizing the media at 121ºC, the media was cooled to 40 ºC. Then, 2% of respective sugars were added to the media. A single colony of yeast was streaked out and the culture media were incubated at 37 ºC. Sugar assimilation abilities of selected yeast isolates were observed daily up to 3 day incubation period.

The ability of anaerobic assimilation (fermentation) of some carbohydrates was determined by using peptone water broth (20 g Peptone and 5g NaCl/l). After sterilizing the media, bromocresol purple solution was added as indicated. 10 ml of peptone water broth media was put in each test tube. After cooling to 40◦C, 0.2g of testing carbohydrate was added to the respective test tube aseptically. Then single colony of yeast strain was inoculated into culture broth and then the culture broth was incubated at 37◦C. The result was observed daily up to 7 day incubation period.

Detection of Accumulated Ammonium Concentration of Indophenols Method

Ammonium accumulation of selected four yeasts isolates in nitrogen fixing yeast broth medium supplemented with various carbon sources were determined by the indophenol method.[23] Four yeasts isolate were inoculated in nitrogen fixing yeast broth medium supplemented with various carbon sources and incubated in water-bath shaker. Culture broth was withdrawn daily and centrifuged at 6000 rpm for 15 minutes. Ammonium presence in the supernatant was estimated by indophenol method. Ammonium concentration in culture broth on various carbon sources for each isolate was compared and the best carbon source for each isolate was selected.

Ammonium accumulation of these selected yeasts isolates was also studied in nitrogen free mineral broth medium (NFMM) with their respective best carbon source. Ammonium concentration was also estimated as described above.

Estimation of Ammonium Accumulation by Viscolor Ammonium Test Kit

Excreted ammonium concentration by isolating strains was estimated using the Viscolor Alpha Ammonium Detection Kit (Macherey-Nagel). A single colony of yeast isolates were incubated in nitrogen fixing yeast broth medium and nitrogen free mineral broth medium for 7 days. After 7 days incubation, culture broth was centrifuged and the supernatant (1 ml) was taken. Two drops of NH4-1 were added to the sample and mixed well, after which one-fifth of a spoonful of NH4-2 was added. After mixing well, the sample was left at room temperature (RT) for 5 min. One drop of NH4-3 was added, mixed well and left at RT for 5 min.

The color development of the supernatants was observed and the ammonium concentration was recorded by comparing with the color chart from the Viscolor Alpha Ammonium Detection Kit.[24]

Pot Trial Study of Nitrogen Fixing Yeast Isolates in Sorghum Plants

For pot trial study in sorghum plant, Sorghum seeds were surface sterilized with sodium hypochlorite (4%) for 5 minutes and then thoroughly rinsed twice with sterile water. The seeds were then soaked in respective broth of yeast isolates for 10 hours.

The inoculated seeds were sown in pots at three seeds per pot in ten replications. After germination, thinning was done to retain only one plant in each pot. The pots were watered regularly to maintain optimum moisture. Each plant was inoculated with 10ml broth (1.4 of OD value) in every 10 days. Un-inoculated plants were used as control in order to study the effect of soil yeasts on the growth of sorghum plants and to compare the nitrogen contents with inoculated treatments.

The sorghum plants were harvested at 45 days after sowing. Plant samples were dried until the dry weight became constant. Dry plant materials were ground into a fine powder. Total nitrogen content in each sample was measured by Kjeldahl method.[25]

RESULTS AND DISCUSSION

Isolation and Selection of Strains

After thirty yeast strains had been isolated from various soil sources, they were screened on their nitrogen fixing activities by culturing on nitrogen fixing yeast medium. Among these isolates, N3, N18, N21 and N24 could grow well on this nitrogen free medium as shown in Figure 1. So, it can be assumed that these four yeasts isolates have nitrogen fixing activity, and they were selected.

Identification of Nitrogen Fixing Yeast Isolate

The morphological characteristics of selected yeast isolates were studied on PYG medium, nutrient medium and nitrogen fixing yeast medium. To the study of morphological characteristics, their colonial color and microscopic morphology were mostly similar although there were some variations in microscopic morphology.

Their sugar assimilation and fermentation abilities were also almost the same and it was described in Table 1. In sugar assimilation, all four isolates could assimilate all tasted sugar although their abilities were different in the assimilation of lactose, Arabians and Myo-inositol. In sugar fermentation, their characteristics were the same on all tested sugars except in maltose and xylose.

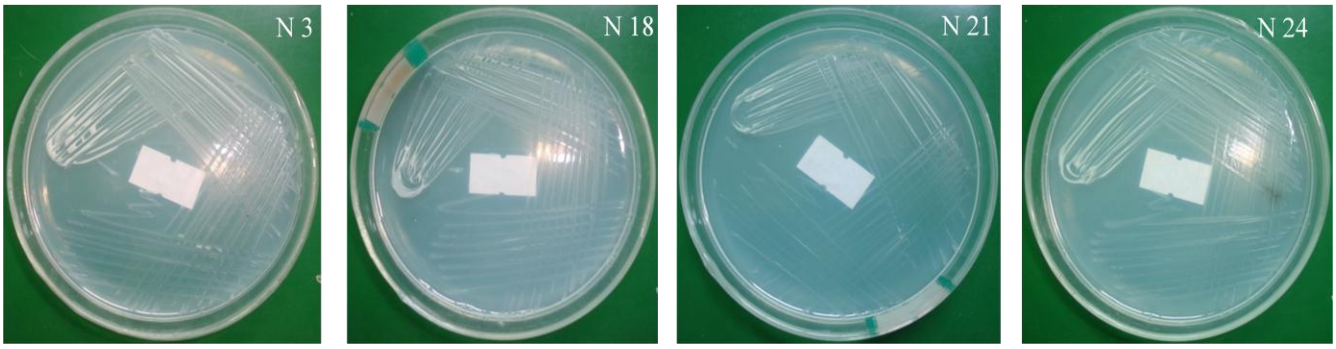

Figure 1: Growth of soil yeast isolates on nitrogen fixing yeast medium

Table 1: Sugar assimilation and fermentation abilities of nitrogen fixing yeast isolate

Sugar	Sugar assimilation				Sugar fermentaion			
	N3	N18	N21	N24	N3	N18	N21	N24
Glucose	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+
Lactose	+(w)	-	-	+(w)	-	-	-	-
Dextrose	+	+	+	+	+	+	+	+
Maltose	+	+	+(w)	+	+	+	-	-
Xylose	+	+	+(w)	+	+	+	-	+
Fructose	+	+	+(w)	+	+	+	+	+
Raffinose	+	+	+	+	+	+	+	+
Arabinose	+(w)	+(w)	-	+	-	-	-	-
Myo-inositol	+(w)	-	-	+(w)	-	-	-	-
Mannitol	+	+	+	+	+	+	+	+

+ (w) = weak growth

Detection of Accumulated Ammonium Concentration of Indophenol Method

Accumulated ammonium concentration of the selected yeast isolates in nitrogen fixing yeast medium with various carbon sources was determined by indophenol method and the results were described in Figure 2. Accumulated ammonium concentrations of yeast isolate were different depending on the carbon sources. The highest quantity of ammonium was accumulated in raffinose and mannitol. Accumulated ammonium concentrations were very low in other carbon

sources containing medium. According to this study, carbon sources significantly effect on ammonium accumulation of soil yeasts.

Then, the selected yeast isolates were also detected on their ammonium accumulation using nitrogen free mineral broth medium with their respective best carbon sources and the results were stated in Figure 3. This medium was commonly used to study nitrogen fixing activities of bacterial isolates. When culture medium was changed although the same carbon sources were used, these isolates accumulated higher amount of ammonium concentration. Inoculation of soil yeast in NFMM broth was enhanced their ammonium accumulation in culture broth.

Therefore, ammonium accumulating activities of selected yeast isolates were different depending on carbon sources and media composition. According to this study, it can be assumed that there are soil yeasts that can accumulate ammonium concentration depending on carbon source.

There are some reports for nitrogen fixation activity of yeast. Millbank reported that no fixation was observed in any of the organisms and the ability of any eukaryote cell to fix nitrogen is doubted.[26] However, Roberts and Wilson confirmed that soil yeasts are capable of taking up molecular nitrogen.[27] Moreover, Charles also reported that organisms including yeasts, pseudo yeasts and molds, tested nearly all show a more or less pronounced power of fixing atmospheric nitrogen.[28]

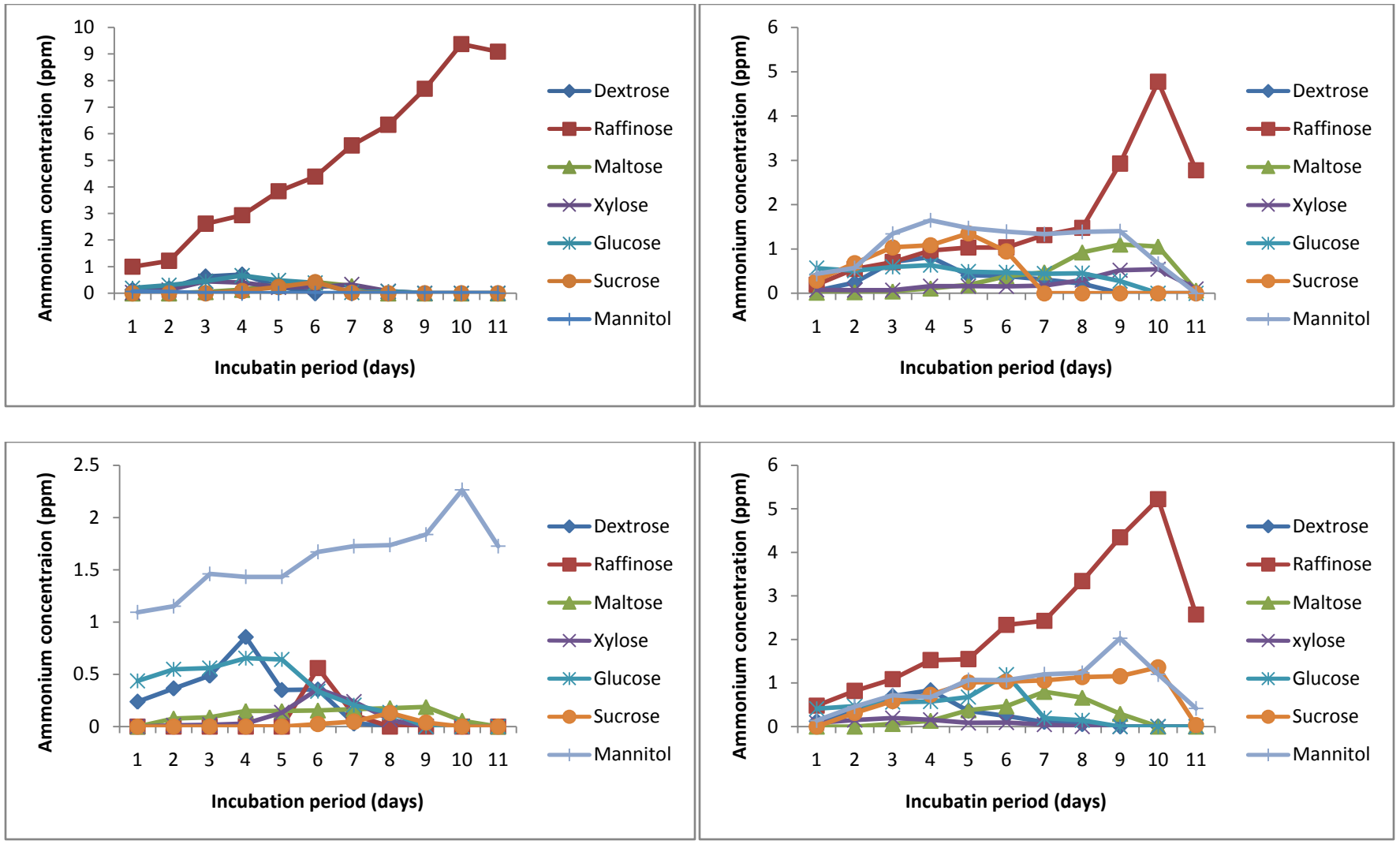

Figure 2: Ammonium Concentration Accumulated by Selected Yeast Isolates in NFYM Broth Using Various Carbon Sources

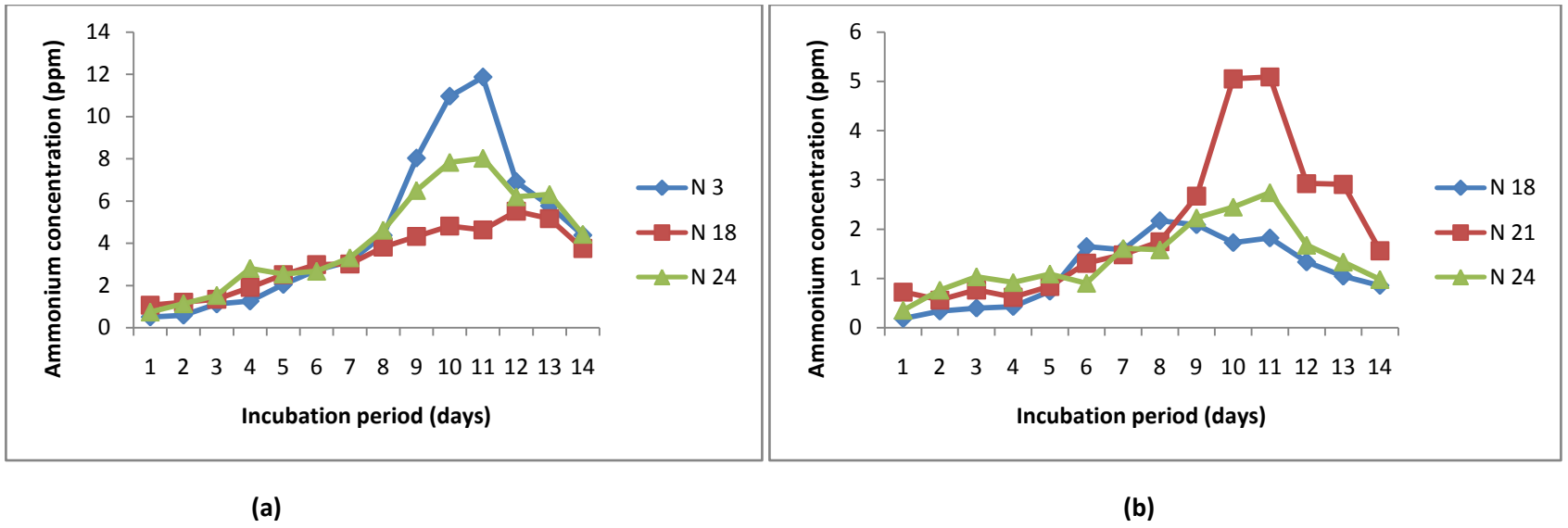

Figure 3: Ammonium Concentration Accumulated by Yeast Isolates in (a) Raffinose-Nitrogen Free Mineral Broth Medium and (b) Mannitol-Nitrogen Free Mineral Broth Medium

Estimation of Ammonium Accumulation by Viscolor Ammonium Detection Kit

Ammonium accumulation of soil yeast isolates were also detected in two different broth media with their respective best carbon source by Viscolor Ammonium Detection Kit (Table 2 and Figure 4). N3 and N24 accumulated above 3ppm of ammonium concentration in broth medium supplemented with raffinose. N21 accumulated above 3ppm of ammonium concentration in mannitol containing NFMM and N18 accumulated highest in raffinose containing NFMM. So, it can confirm that MFMM media is more suitable for ammonium accumulation.

Table 2: Estimation of Accumulated Ammonium Concentration by Selected Yeast Isolates using Ammonium Test Kit

Yeast Isolates	Ammonium concentration in two different broth media (mg/l)			
	NFYM (mannitol)	NFMM (mannitol)	NFYM (raffinsoe)	NFMM (raffinose)
N3	0-0.2	0-0.2	>3	>3
N18	0.2-0.5	2- 3	2- 3	>3
N21	2-3	> 3	0.2-0.5	1-2
N24	0.5-1	2-3	>3	>3

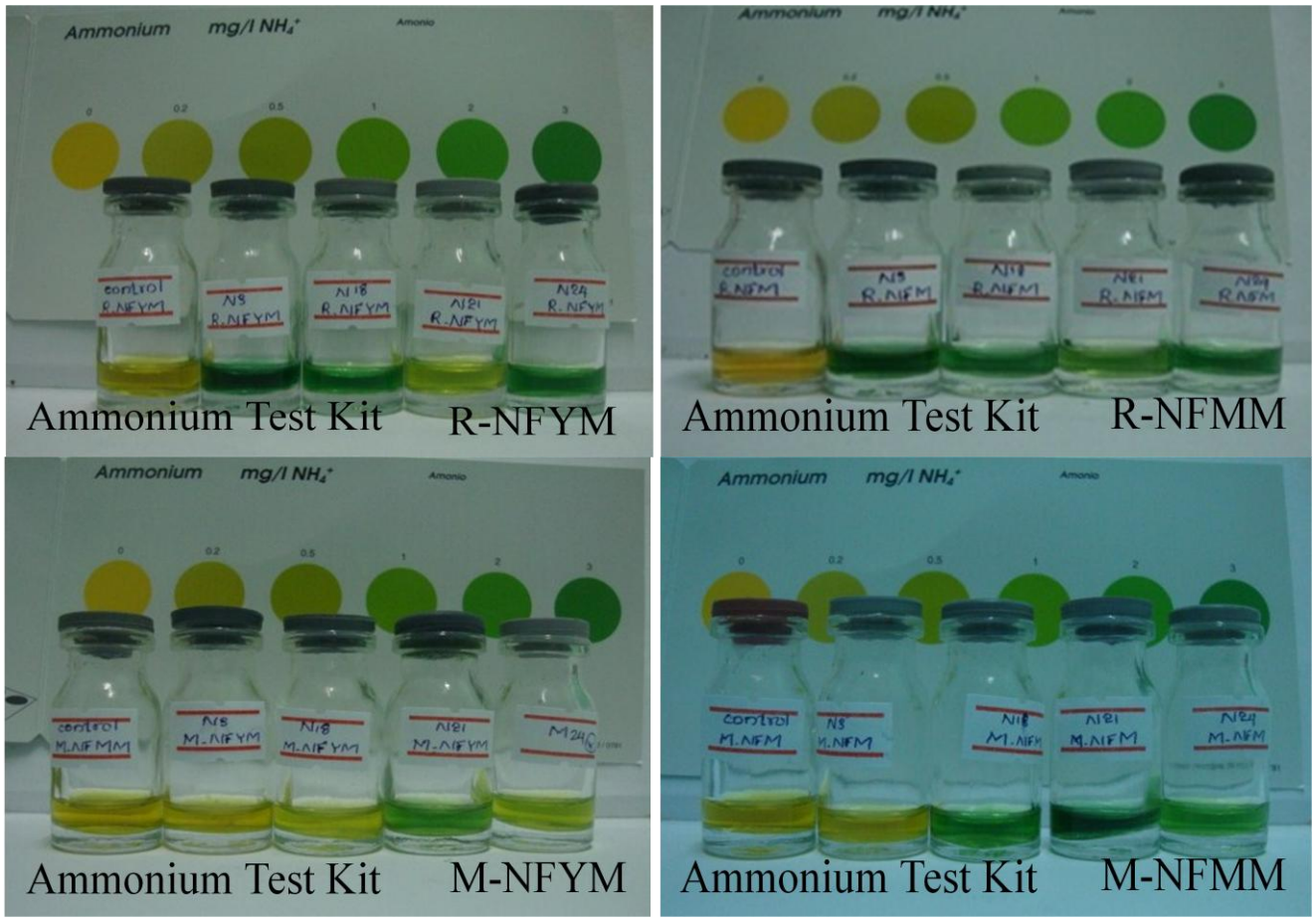

Figure 4: Amount of Accumulated Ammonium Concentration of Selected Yeast Isolates

Pot Trial Study of Nitrogen Fixing Yeast Isolates in Sorghum Plants

In this pot culture experiment, total nitrogen contents in plant samples of all treatments were shown in Table 3. The inoculation of three selected nitrogen fixing yeast strains increased total nitrogen contents of plant over control treatments. Among them, N3 and N24 were best by giving higher nitrogen content of plants. N3 and N24 accumulated higher amount of ammonium concentration than other two strains. It was also found that two strains (N3 and N24) gave highest total nitrogen content in sorghum plants. So, the ammonium accumulating activity of soil yeasts directly affects on plant growth and total nitrogen content.

Searching new yeasts as bio-fertilizers and studying their productivity of bioactive chemical compounds expand our knowledge about their approached mechanisms to enhance the plant growth and soil characteristics (Agamy 2013).[20]

The positive effect of yeast is supported by the findings of Mekki and Ahmed.[29] They stated that the increase in yield components because of yeast treatment is mainly attributed to the effect of yeast, which can play a very significant role in making available nutrient elements for plants. In addition, yeast content of macro and micronutrients, growth regulators and vitamins stimulate the plant to build up dry matters.[30, 31]

Table 3: Total Nitrogen Contents of Sorghum Plants at 45 Days after Sowing

Treatments	Total nitrogen content (%)
T1 (N3)	1.52
T2 (N18)	1.25
T3 (N21)	1.33
T4 (N24)	1.51
T5 (water)	1.14
T6 (Nitrogen Fixing Yeast Media)	1.16
T7 (urea)	1.25

CONCLUSION

Among all isolated yeasts, four yeast isolates were selected as nitrogen fixing yeast strains according to their growth on nitrogen free medium. In the determination of ammonium concentration by indophenol method using eight carbon sources, they accumulated higher ammonium concentration in using mannitol and raffinose as carbon source. The selected yeast isolates accumulated higher ammonium concentration in nitrogen free mineral medium than nitrogen fixing yeast medium.

In a pot trial study of sorghum plants for 45 days, three inoculated treatments gave higher total nitrogen content than control treatments.

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