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Isolation and characterization of microorganisms for potential use with manure and chemical fertilizers

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Abstract

In the present study a microbial consortium with the ability to utilize nitrogen of certain chemical fertilizers were established with a series of tests. The strains' indole acetic acid (IAA) and siderophore production, utilization of urea as carbon source were tested with 15 isolates, %50 of which belonged to *Bacillus* spp. based on caseinase and amylase activities. Nitrogen utilization was tested with nitrogen fixation, ammonia and nitrite oxidation abilities of the strains. Phosphate evaluation, another component of chemical fertilizers, was also determined with phosphate solubility test conducted in the presence of Pikovskaya agar. Identification of isolates was conducted via evaluating MALDI-TOF MS analyses. Results indicated four strains, 3 of which were identified as *Bacillus cereus* sp. and an *Alcaligenes faecalis* sp. which was the only non-*Bacillus* member of the consortium. The members of the consortium showed no antagonistic activity against each other implying their successful utilization as components of a commercial fertilizer. Media selection along with diversified tests were concluded to serve as fine criteria to establish a microbial consortium with targeted features.

Keywords: Indole acetic acid, siderophore, *Bacillus* spp., nitrogen fixation, ammonia/nitrite oxidation

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Organik ve kimyasal gübre olarak kullanım potansiyeline sahip mikroorganizmaların izolasyon ve karakterizasyonu

Özet

Sunulan çalışma kapsamında kimyasal gübre içeriğindeki azotu kullanabilme özelliğine sahip mikroorganizmalardan oluşan bir konsorsiyum bir dizi test sonucunun değerlendirilmesiyle oluşturulmuştur. Suşların indol asetik asit (IAA) ve siderofor üretme yetenekleri ve üre içeriğinde yer alan karbonu besiyeri olarak kullanma yetenekleri 15 farklı izolat için test edilmiştir. Suşlara uygulanan amilaz ve kazeinaz aktivite testleri sonucunda %50'sinin *Bacillus* spp. olduğu belirlenmiştir. Azot kullanımının incelenmesi kapsamında suşların azotu fikse edebilme ayrıca amonyak ve nitriti oksitleme yetenekleri belirlenmiştir. Kimyasal gübrelerde bir diğer makro element olan fosfat kullanımı Pikosvkaya agar'da inokülasyon ile görülmüştür. Son olarak suşların cins bazında tanımlanması MALDI-TOF MS analizleri ile gerçekleştirilmiştir. Sonuçların değerlendirilmesiyle mikroorganizma karışımına üç *Bacillus cereus* sp. suşu ile karışımın *Bacillus* sp. olmayan tek üyesi *Alcaligenes faecalis* sp. seçilmiştir. Mikroorganizma karışımında yer alan suşların birbirlerine karşı antagonistik etki göstermediği dolayısıyla karışımın mikrobiyal gübre olarak ticarileştirilmesinin mümkün olduğu da elde edilen sonuçlar arasındadır. Uygun besi yeri seçimi ve hedefe yönelik testlerin uygulanması hedefe yönelik mikroorganizmalardan oluşan bir konsorsiyumun oluşturulmasında etkili olmuştur.

Anahtar kelimeler: İndol asetik asit, siderofor, *Bacillus* spp. azot fiksasyonu, amonyum/nitrit oksidasyonu

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1. Introduction

The opinion that world population would have risen over 9 billion in the next 40 years is acknowledged by a majority of demographers and environmentalists. The inevitable increase in population resulted in deficit of sources needed to meet nutritional requirement [1]. Proper nutrition of plant to achieve maximum yield and product quality has become a popular research area due to population increase. Addition of chemical fertilizers to soil is the most frequent and privileged application to solve yield and quality problems. Utilization of chemical fertilizers are lower in Türkiye compared to developed and developing countries. Nevertheless, chemical fertilizer is gaining an increasing attention and its harmful effects on environment possesses a serious threat [2].

Nitrogen, phosphorus and potassium are the main constituents of chemical fertilizer and the impact on environment varies according to the element. Nitrogen in the form of nitrate has the potential to accumulate in drinking water and nitrosamine compounds are known to be highly carcinogenic. Heavy metal accumulation is another problem during utilization of chemical fertilizers [2].

The harm oriented from chemical fertilizers is in fact related to its excessive use. Soil with its high buffering potential could tolerate chemical fertilizers to an extent. However, exceeding threshold leads to significant health and environmental problems [3].

Hence studies, mainly focused on developing the substitute of chemical fertilizers, have recently increased. The prerequisite of these substitutes is to lower environmental impact along with maintaining sustainable production. Plant growth promoting bacteria are fine candidates meeting these demands. These bacteria, when added to soil, operate via certain mechanisms facilitating acquisition of already existing resources in soil [4].

Facilitation of existing resources are mostly accomplished through solubilization of phosphate and nitrogen fixation from atmosphere. The compounds related to these two elements exist in high amounts in soil, yet the problem is the deficit of compounds suitable for plant uptake. PGPB intercedes at this point enabling their acquisition by plant. PGPB could also produce cytokinin, gibberellins and indole acetic acid (IAA). These bacteria also operate via indirect mechanisms such as siderophore production. Siderophore producing bacteria also acts as biocontrol agent capable of decreasing phytopathogen population in the flora [4,5].

The number of studies regarding the use of PGPB as fertilizer is relatively low, however, the results are promising with examples of successful utilization in mangrove reforestation [6], development of faba bean seeds [7] domestic apple [8].

Based on the findings presented so far, it was our opinion that PGPB could have been successful in the case of either simultaneous addition with chemical fertilizer or application to soil already containing chemical fertilizer. Apart from nitrogen which could be fixed via PGPB from atmosphere, PGPB would be less effective in the case of a serious nutrient deficit in soil. Hence the main purpose of PGPB utilization should be enhancement of nutrient uptake which was supplied via chemical fertilization or by other means. Chemical fertilizer is highly effective over a short period of time. However, long-term utilization of chemical fertilizer requires its simultaneous application with organic matter such as manure and straw.

Certain procedures were developed and/or modified in the present study to establish a microbial consortium. The procedures were developed to select plant growth-promoting microorganisms. These were intended to act as fertilizer in the case of sole utilization and potential enhancer when utilized in conjunction with either manure or chemical fertilizer.

2. Materials and methods

2.1. Semi-selective isolation procedure as starting-point

As previously stated, the priority in selection of consortium members is the ability to utilize nitrogen supplied by any kind of chemical fertilizers. Selection of consortium members started with semi-selective isolation procedure from 18-month compost samples. These samples were previously obtained from compost prepared by manure, wheat straw and grass. Semi-selective isolation procedures were simply consecutive dilution of 0.5 g samples and inoculation of 10^{-6} dilutions in an ammonifying medium containing 0.05% peptone, 0.05% KH_2PO_4, 0.05% K_2HPO_4, 0.05% $MgSO_4.7H_2O$ in 1 L with 12 g agar. This medium was recently utilized for enumeration of ammonifying bacteria [9]. The media was adopted as isolation procedure and acted as a starting point to determine microorganisms with the ability to utilize organic nitrogen. Isolation procedure was followed by purification and obtaining isolates as single colonies. These isolates were further tested for their potential as PGPB.

2.2. Standard tests applied to determine the potential of isolates as PGPB.

Nitrogen fixation ability of selected isolates was initially tested to detect isolates with ability to fix atmospheric nitrogen. Traditional procedure with semi-solid agar require long-term inoculation, instead a simpler and practical method based on inoculation with Ashby's mannitol and Ashby's sucrose agar was adopted to determine isolates with nitrogen fixing ability. Ashby's agars were originally utilized to isolate nitrogen fixing microorganism. Inoculation in the presence

of these agars were simply utilized as test procedure in the present study. Ashby's agar contained (g/L) 0.2 KH_2PO_4, 0.2 $MgSO_4.7H_2O$, 0.2 NaCl, 0.1 K_2SO_4, 5$CaCO_3$ and 15 agar. Carbon sources were mannitol and sucrose which were added 20 g/L [10].

IAA production was determined with color change to pink at 530 nm via UV-Vis spectrophotometer. The method contains addition of Salkowski reagent to 1 ml of Luria Bertani supernatant containing % 0.05 l-tryptophan. 1.35 g $FeCl_3.6H_2O$ was completed to 100 ml via dH_2O and 2 ml of this solution was then combined with 49 ml dH_2O and 49 ml of per chloric acid, respectively. Salkowski reagent was added with the ratio of 2/1. The strains were incubated at 30 ℃ for 48 hours in l-tryptophan containing LB broth [11].

Pikovskaya agar containing (g/L) 0.5 yeast extract, 10 dextrose, 5 Ca_3PO_4, 0.5 $(NH_4)_2SO_4$, 0.2 KCl, 0.1 $MgSO_4$, 0.0001 $MnSO_4$, 0.0001 $FeSO_4$ and 15 agar were utilized to determine phosphate solubility. Transparent zone formation was evaluated as positive indicator [12].

Chrome Azurol S Assay with certain modifications was applied to determine siderophore production of strains. CAS solution prepared with mixing of 4 solutions was finally mixed with King's B agar to formulate CAS agar. The ratio of CAS solution to Kings's B agar was adjusted to 35/100 according to the method [13]. The ingredients of CAS solution and King's B agar were given in Table 1.

Table 1. Chrome Azurol S agar utilized in siderophore production

Solutions				
C	**D**	**B**	**A**	**King's B agar (g/L)**
0.03 ml HCl, complete to 100 ml via dH_2O	0.026 g $FeCl_3.6H_2O$, complete to 100 ml via dH_2O	0.06 g Chrome Azurol reagent, complete to 50 ml via dH_2O	0.07 g HDTMA, complete to 40ml via dH_2O	Peptone 20 $MgSO_4.7H_2O$ 1.5 KH_2PO_4 1.5 Glycerine 10 ml Agar 20
Add 10 ml	Add 100 ml	Add 50 ml	Add 40 ml	pH 7.2
Procedure:				
Solutions were combined with the order of C, D, B and A. The resulting 200 ml solution was finally mixed with King's B agar with the ratio of 35/100				

Urea degradation ability of strains was tested via urea broth. The test was based on strains' urea utilization as carbon source. Ammonium formation during the process was detected by color change of the broth. Inoculation of strains was conducted at 37 ℃ for 48 hours. The original "orange red" color of the broth turned into red in the presence of strains with positive results. The broth utilized in the study contained (g/L) 0.1 yeast extract, 9.1 KH_2PO_4, 9.5 Na_2HPO_4, 20 urea and 0.01 phenol red.

2.3. Specified tests to determine nitrogen evaluation potential of PGP isolates.

The highlight of the present study was to determine isolates with the ability to facilitate plant's nitrogen utilization. Hence sustainable supplement of plant available nitrate nitrogen was crucial for crop development. As previously stated the isolates were examined to detect the ones with the ability to transform ammonia from organic nitrogen and urea. However, these are only the initial states of nitrogen cycle and nitrogen should be converted to nitrate. Hence two media originally utilized for enumeration of both ammonium and nitride oxidizers were utilized to test nitrifying potential of strains. The media utilized for ammonia oxidation contained (g/L) 0.5 $(NH_4)_2SO_4$, 1 K_2HPO_4, 0.03 $FeSO_4.7H_2O$, 0.3 NaCl, 0.3 $MgSO_4.7H_2O$, 7.5 $CaCO_3$. Nitride oxidizing potential of strains was determined via of a separate medium containing (g/L) 0.006 $NaNO_3$ as nitrogen source. The media which was originally used for ammonia oxidizer detection was further modified with $CaCl_2$ addition. Calcium was supplied with 0.1 $CaCl_2$ and 1 $CaCO_3$ in nitride oxidizing media. The remaining ingredients and their amounts were kept identical. Nitrifying potential of strains was determined based on color change. Magenta colored media formed via phenol addition and pH adjustment to 8 should change into shades of yellow due to a decrease in pH value [14].

2.4. Biochemical tests conducted to determine Bacillus sp. in accordance with MALDI-TOF MS analyses evaluated for validation.

Biochemical tests were selected based on facile determination of *Bacillus* sp. presence among isolates. Evaluation of tests were based on positive results for both caseinase activity and starch hydrolysis. The modified medium used for caseinase activity contains (g/L) 5 peptone, 3 meat extract, and 15 agar. The ingredients were dissolved in 900

ml dH_2O. The procedure was modified with addition of %10 skim milk after sterilization. Zone formation was the expected indicator for caseinase activity [15].

Starch hydrolysis was also evaluated according to zone formation following Lugol addition to the agar plates prepared with (g/L) 5 peptone, 3 meat extract, 2 starch and 15 agar. The test was conducted at 37 ℃ for 24 h [16].
The tests applied in the course of study were simply inoculation procedures conducted to visualize zone or color change in media. All agar inoculation was applied at 30 ℃ unless stated otherwise. The last test applied to validate *Bacillus* sp. presence was MALDI-TOF MS analyses applied for selected strains and an isolate with positive activity towards siderophore production (See. Results and Discussion). MALDI-TOF MS analyses were conducted via service procurement from Hatay Mustafa Kemal University.

3. Results

Variation of IAA production capacity was illustrated in Figure 1. The values were obtained for 3 separate runs and capacity values were presented as pie chart with clockwise variation starting from LFSN1 isolate. In our opinion, the capability of IAA production was more important compared to the amount of IAA produced as IAA production was adopted as a standard criterion for selection. Hence the figure was evaluated for the strains with the highest production capacity, yet remaining tests were applied for all.

Nitrogen fixation from atmosphere was another important property that should be standard for a microbial consortium. The consortium was originally combined to facilitate plants' fertilizer consumption, however properties such as IAA production and nitrogen fixation ability were also crucial in the case of fertilizer deficit. Inoculation performances of isolates in the presence of Ashby's sucrose and mannitol agars (AMA and ASA) were illustrated in Table 2. Results indicated relatively higher affinity of the isolates towards mannitol presence.

Table 2. Nitrogen fixation performance of isolates in the presence of AMA and ASA agars; biochemical tests to determine caseinase activity and starch hydrolysis

	Nitrogen fixation		Biochemical tests	
Isolate code	**AMA**	**ASA**	**Caseinase**	**Starch hydrolysis**
LFNS1	++	+	-	-
LFNS2	++	+	+	+
LFNS3	++	+	-	-
LFNS4	--	+	-	-
LFNS5	+	+	+	+
LFNS6	+	++	+	+
LFNS7	+	+	+	+
LFNS8	--	+	+	+
LFNS9	++	+	+	+
LFNS10	++	+	-	-
LFNS11	+	+	+	+
LFNS12	++	+	-	-
LFNS13	--	--	-	-
LFNS14	--	--	+	+
LFNS15	+	+	-	-

+: production with visible colonies
++: completed development of isolates, stronger production

As previously stated, a semi-selective agar was adopted during isolation of microorganisms. The idea was to detect certain microorganisms preferably the ones with ability to utilize organic nitrogen. A simple twist to the procedure was conducted with exclusion of agar from the media and following addition of Nessler's reagent. By doing so, the isolation procedure could easily be evaluated for the selection of ammonifiers. The results for both agar and broth were given in Figure 2. The isolates with positive results were given in Figures with orange color formation as positive indicators.

Phosphate solubility in the presence of Pikovskaya's agar could be determined via transparent zone formation. Results given in comparison with a positive sample revealed only 3 isolates with the ability to solubilize inorganic phosphate. Hence this test has become one of the main selection criterion in the course of microbial consortium preparation (Figure 3).

Siderophore synthesis to facilitate iron transport to plants was another selection criterion. The results indicated only LFNS13 as siderophore producer (Figure 4).

Urea degradation ability of strains was initially conducted as specified test. The test determines degradation of urea and hence production of ammonia through urea utilization as carbon source. Figure 5 revealed the color change from orange-red to red color for LFNS6 and LFNS15 isolates.

Ammonium oxidation and nitrite oxidation ability of the strains were also determined via color change from magenta to yellow color. Results were illustrated in Figure 6.

4. Conclusions and discussion

Selection of isolates as participants of a microbial consortium might as well be an impossible task considering similar properties that the isolates might possess. Isolation procedures are generally conducted at identical conditions including media, inoculation temperature and antibiotics utilization if required. Hence a different approach meeting multiple criteria should be adopted during isolation. The isolation procedure itself becomes a selection criterion with the use of various antibiotics and heating procedures. However, antibiotics utilization is expensive and there is no guarantee for acquisition of isolates with beneficial traits for plant growth. Hence a more facile method based on media selection was applied in the present study. As previously stated, the isolation procedure is originally utilized for determination of ammonifiers among isolates. Isolates with the ability to mineralize organic nitrogen is in fact not preferred in the case of composting. On the contrary, studies on composting aim to increase bioavailable organic nitrogen content during the process and regulation of nitrogen conversion in the course of composting is considered as the key factor [17]. However, the conditions in the present study were entirely different with mature compost being the source of isolation and isolates with the ability of utilizing organic nitrogen would be preferable in the case of manure application to cultivation area. In other words, isolates using organic nitrogen would continue to survive or even thrive long after their application to cultivation area amended with manure.

Nitrogen fixation from atmosphere was considered as another solution for survival of potential consortium. This was considered as the worst-case scenario for cultivation areas without amendment of any kind. It is noteworthy to point out the fact that the medium AMA and ASA could both be used as selective isolation media and in that case isolates with the ability to fix atmospheric nitrogen would be starting point for establishing the consortium.

Nitrogen utilization of isolates was one of the key factors during selection of isolates. Another key factor of selection was the ability of isolate to utilize the fertilizer as media. This scenario was tested with urea degradation in which urea was the carbon source. Urea is preferred among the farmers as the fertilizer continues the highest among of nitrogen with lowest price. The main problem in urea utilization ammonia volatilization. Certain precautions to reduce ammonia emissions are still being studied. However, these precautions generally included utilization of additives to reduce ammonia leading to an increase in the amount of externally supplied chemicals to the soil. Utilization of microbial consortium offers an accelerated pathway with utilization of urea as food source. Urea degradation will result in ammonia volatilization. However, enhanced breakdown of urea to ammonia will also lead to plant available ammonium formation in the presence of water. Consequently, it was thought that addition of urea degradaing isolates to the consortium would have increased the amount of ammonia transforming to ammonium. The reactions occurred during ammonium formation was given as [18]:

$$(NH_2)_2CO + H_2O \longrightarrow NH_3 + H_2NCOOH \longrightarrow 2NH_{3\,(gas)} + CO_{2\,(gas)} \quad (1)$$

$$NH_3 + H_2O \longrightarrow NH_4^+ + OH^- \quad (2)$$

Microbial consortium would also contain isolates with the ability to transform nitrogen into plant available nitrate through ammonia and nitrite oxidation mechanisms. Tranformation of ammonia or ammonium to nitrate is a two step process involving initial formation of nitrite which is then converted to nitrate. It was crucial to include both ammonium and nitrite oxidizers to the consortium to maintain plants' highest possible nitrate assimilation [19]. The aim was simply to determine the potential of isolates that could oxidize ammonium/ammonia and/or nitrite to nitrate. Hence observation of color change through inoculation in ammonia and nitrite oxidizer in the presence of media utilized for enamuration was thought to be a simple yet an effective method to determine isolates. As long as an isolate could be determined based on the tests conducted so far, the consortium would be effective as the isolates would be able to directly interfere to nitrogen cycle, hence increasing plants' nitrate assimilation.

Phosphorus, another macronutrient for plant growth, is scarce which impels its external addition. Another problem in utilization of phosphorus is the amount of $CaCO_3$ in Türkiye. 48 % of agricultural area in Türkiye was classified as mid-calcitic while there is a surplus of $CaCO_3$ in 30 % of Türkiye's agricultural area [20]. Phosphorus when added to soil reacts with $CaCO_3$ which resulted in formation of Ca_3PO_4 unavialble for plant assimilation. External phosphorus application is a continious process. In other words there is a vast and increasing source of potassium in agricultural area. Selected isolates would be effective at this point dissolving Ca_3PO_4 which will result in formation of plant available phosphorus [21]. Iron deficiency is also closely related to $CaCO_3$ presence and an isolate with the ability of producing siderophore will benefit from the presence of a phosphate solubilizing isolate when applied in accordance [22].

Biochemical tests were also applied in the course of study to maintain uniformity of the isolates within the bounds of possibility. Results obtained from caseinase actvity and strach hydrolysis revealed the first clue on determination of isolates. Hence over 50 % of isolates could hydrolyse starch and have caseinase activity and had a very high chance to belong *Bacillus* spp. (Table 2). Based on the results a scheme revealing the effects of isolates was prepared and illustrated in Figure 7.

Selection of consortium members is primarily detected according to the results of specific tests. Some results were unique for certain *Bacillus* sp. members and hence these strains were directly added to the consortium. Urea degradation was one of the tests with specific results and LFNS6 was the only strain with positive results. This is not the only property of the strain, LFNS6 could also utilize atmospheric and organic nitrogen. This strain also had the ability to oxidize both ammonium and nitrite which makes it a perfect candidate for use in consortium. Another strain LFNS8 could also utilize organic nitrogen. The strain further oxidizes ammonium to nitrite and nitrite to nitrate. Apart from these properties the strain's distinguishing feature and reason of selection was its ability to solubilize phosphate.

LFNS9 was selected with its features similar to LFNS8 with an additive feature of nitrogen fixing ability. Except siderophore production, this strain with its many properties was an ideal candidate for consortium. LFNS13 was the only non-*Bacillus* member of the consortium and selected for its unique feature as siderophore producer. Hence microbial consortium included LFNS6, LFNS8, LFNS9 and LFNS13.

Determination of antagonism between the strains was conducted with a relatively easy procedure. This test was among the simplest procedures, yet the results were crucial in case the consortium would be commercialized as bio fertilizer. The results for consortium including LFNS6,8,9 and 13 were illustrated in Figure 8. Consortium members could thrive in same medium as seen from the figure (Figure 8).

The last step in determination of microbial consortium was the identification of members. Based on the results, selected members of *Bacillus* spp. along with LFNS13 were analyzed via MALDI-TOF MS device. The results for selected consortium members were given in Table 3. Score values for LFNS6, 8 and 9 were between 2-2.299 revealing selection of the right genus. However, identification of species with these scores also revealed a probability implying the presence of members other than *Bacillus cereus* sp. LFNS13 was thought to be *Alcaligenes faecalis* sp. based on the results.

Table 3. MALDI-TOF MS analysis results for selected *Bacillus* sp. strains

Analyte code	**Organism (best match)**	**Score value**
LFNS6	*Bacillus cereus*	1.846
LFNS8	*Bacillus cereus*	2.03
LFNS9	*Bacillus cereus*	2.092
LFNS13	*Alcalinegenes faecalis*	2.529

MALDI-TOF MS analysis is more viable in terms of economy when compared with 16S rRNA sequencing. The results obtained for *Bacillus cereus* sp. is a unique situation and it was thought that identical results would have been acquired via 16S rRNA sequencing. 16S rRNA sequencing is a costlier alternative and results indicated that MALDI-TOF MS should have been considered as the first step prior to trying costlier alternatives.

As stated, the score values for *Bacillus cereus* sp. was unique yet these values should be expected in this particular case. *Bacillus cereus* was indeed a group of organisms with four members which were recently identified. The members *Bacillus cereus, Bacillus thuringiensis, Bacillus mycoidos* and *Bacillus anthracis* could only be separated with few physiological characteristics [23]. Literature survey on *Bacillus cereus* sp. indicated the presence of various strains including siderophore producing [24] members. Consequently, an advanced analysis such as whole genome sequencing was required for detailed validation.

LFNS13 identified as *Alcalinegenes faecalis* sp. is one of the well-known organisms studied for its plant growth promoting traits. A quick literature survey on the species revealed phosphate solubilizing ability of some strains [25]. However, that was not the case for our strain and the only selection criterion for LFNS13 was its siderophore production ability which was unique among other isolates. The conclusions obtained in the present study were summarized below.

- Media selection could serve as a selection criterion to assemble a certain group of microorganisms.
- Tests conducted for isolates could be diversified to establish a consortium with modified properties.
- Test procedures could be modified to facilitate establishment of microbial consortium.
- Strains with different properties could serve as members of consortium which was the case in our study.
- Identification in species level was possible to a certain extent. MALDI-TOF MS analysis presented a brief idea on strains and similar results would have been obtained in the case of applying its costlier alternatives. Hence a relatively simpler procedure is concluded to be better for initial investigation.

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