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RESEARCH ARTICLE

POTENTIAL of ENZYMATIC METHODS and BIOLOG ECOPLATE ANALYSIS for INVESTIGATION of MICROBIAL FUNCTIONALITY in AGRICULTURAL SOILS

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ABSTRACT

Agricultural systems and applications affects the soil ecological conditions and microbial structure. However, it is important that the interventions do not disturb the balance and quality of the microbial content of the soil. For this reason, enzymatic methods and Biolog Ecoplate method were applied to research and evaluate the status of functional microbial diversity in four different agricultural soil samples (A, B, C, D).

The results indicated that the pH, electrical conductivity, amounts of total nitrogen (N) and humidity were generally similar but calcium carbonate rates were higher in A and B agricultural soils. Soil enzymatic activity results showed some differences among the four different agricultural soils. The activity measurements of urease, phosphatase and dehydrogenase were high and results showed the differanties. Enzymatic activities and microbial populations correlated with each others and content of organic carbon.

Evaluation of substrate utilization profiles and the diversity indices concluded that microbial community structure and composition were different related to various conditions. The average well color development (AWCD) which was calculated in the Biolog EcoPlate analysis showed some variations in the catabolic ability of four different agricultural soil samples' microbial communities. Compared to other samples, C and D agricultural soil samples had a higher overall AWCD value. AWCD of C soil was significantly higher than in the others. Lowest used substrates were α-Cyclodextrin, α-Ketobutyric acid, β-Methyl-D-glucoside, α-D-Lactose and 2- hydroxybutyric acid. The most extensively used substrates were aminoacids and carbohydrate groups. These results indicates the degradation potential. With the Biolog EcoPlate and enzymatic measurements, changes in the microbial community in agricultural soils can be detected, and also agricultural management and application methods for tillage can be evaluated.

Keywords: *Dehydrogenase, urease, phosphatase, AWCD, Biolog EcoPlate, microbial activity*

JSR A
Journal of Scientific Reports

1. INTRODUCTION

Soil microorganisms have important roles in most of soil reactions such as organic matter formation, decomposition, respiration and nutrient cycle [1, 2]. The physical and chemical properties of the soil changes quite slowly, but the biodiversity and biochemical reactions in the soil microflora are affected very quickly by changes and give a rapid reaction so they are sensitive reactions and signatures for evaluating soil quality [3, 4]. Soil microbial communities differ metabolically and genetically in response to different applications in the organic farming practices. The microbial structure of the soil is very critical for the ecosystem to create sustainable plant communities based on quality soil structure and biological interactions [5]. Also protection of the microbial activity and diversity in the soil is very important for sustainable agriculture management [6]. Many agricultural applications such as different soil managements and tillage techniques cause to modifications in size structure and biological reactions of soil microbiota [7, 8]. Organic modification of agricultural systems is thought to increase soil quality, biologically and chemically [9]. Organic and inorganic substances are applied for supporting nutrient intake to plants [10]. Organic substances in the soil stimulate plant growth and affect the physical properties of the soil [11]. However organic and inorganic substances can cause to changes in the population structure, composition, physiology and ecology of soil microorganisms [10]. For example, balanced use of key elements (N, P, K) in fertilization can be beneficial for efficient plant growth, but farmers follow an economic strategy rather than an agricultural strategy. This creates negative consequences. Unbalanced fertilization is common, but data on the effect of this process on soil microbial community is limited [12]. Changes in factors such as the type, quality, seasonal distribution, nutrient inputs of plant residues, rotation in the soil during agricultural production affect microbial structure, density, diversity and processes [13]. The response and change of microorganisms to different applications such as pedoclimate and crop rotation are still not fully understood. The density of different microorganism groups is also an indicator of the changes that occur in the soil ecosystem when different plants are cultivated. Nutrient cycle is very important for plant production efficiency in crop rotation applications. Soil microorganisms and soil enzymes take part in the nutrient cycle [14]. In addition, studies on chemical and biological properties of different agricultural soils are unkonown [15].

Microbial community analysis in soils can be detected with many different methods. Their effected rate can be detected with the determination of changes in different reactions, such as soil respiration, metabolic activity, microbial biomass, cultural and direct microscopic countings, chloroform fumigation incubation, ATP measurements, total amount of phospholipid fatty acids, Biolog and molecular analysis [6, 16].

However, most of the methods are not completely accurate and determination of microbial diversity also does not reflect functionality, as most of the microorganisms in the soil are in rest and inactive phases. The Community Level Physiological Profile (CLPP) of soil bacteria is effective and pioneering method which analysis soil quality [17]. Analysis based on the use of carbon resources is essential in this method and detect functional diversity changes of soil microorganisms [16]. Using the CLPP method, the effects of soil additives can be evaluated by examining the changes in the activity and diversity of microorganisms in the soil [18, 19]. CLPP generates vast amounts of data that are extremely difficult and complex to interpret. Biolog-generated data include the AverageWell Colour Development (AWCD), the Shannon diversity index (H), substrate richness (SR) and Shannon

evenness (E) for analysis. AWCD values indicates the microbial community's potential metabolic activity. The Shannon diversity index (H) is used for the calculation of the physiological diversity of bacterial communities. The Biolog EcoPlate can be used in effects of heavy metals, hydrocarbons, estimating the impact of stress factors (salinity, pH) [19].

Using of soil enzymes to search and examine soil biological activity is another method [20]. Usually for this purpose in studies monitoring of β-glucosidase, dehydrogenase and urease activities is used. β-Glucosidase is a hydrolytic enzyme and urease is responsible for providing the nitrogen to plants. These enzymes indicate the soil quality. Dehydrogenase is an oxidoreductase and can be used in determination of soil microbial activity [21-23].

Therefore, in this study, it was aimed to evaluate different agricultural soils and reveal how catabolic diversity, microbial structure, composition and function were changed at the community level. For these purposes, enzymatic methods and Biolog EcoPlate were used for microbiota activity and functionality.

2. MATERIALS and METHODS

2.1. Field Sampling

Four agricultural soil samples (A, B, C, D) from lands which coordinates were near the 39°59'43.14" N, 32°20'39.09" S in Ayaş, Ankara (Figure 1) were collected on April 2019 from 20 cm depth using a soil auger. Collected soil samples were brought to the laboratory for physicochemical, microbiological, and biochemical analysis. The soils were then stored at 4 °C. For analysis, agricultural soil samples were used by sieving and air drying. For microbiological and biochemical analysis of soils, moist soils were used.

Figure 1. The study area on the map and sampling point.

JSR A
Journal of Scientific Reports

2.2. Physico-Chemical Soil Properties
The pH value of the agricultural soil samples were determined with the HACH HQ40D multi-parameter pH meter and their electrical conductivity were detected with the HACH HQ40D multi-parameter EC meter. A total of 10 g of soil and 10 ml of distilled water was stirred with a vortex mixer in a centrifuge tube and then a pH was measured [24]. Then, electrical conductivity (EC) was measured with 1:2.5 of soil:water slurry.

2.3. Calcium Carbonate Analysis
Scheibler calcimeter was used for carbonate determination by volumetric method. For this, 1 g dried agricultural soil samples were weighed and put into 25 ml conical flasks. 1/3 of HCl was filled into an acid tube and carefully put into the erlenmeyer with the help of pliers without pouring. The pure water level of the calcimeter was set to zero and the mouth of the flask was closed with the rubber stopper on the calcimeter, and the acid in the erlenmeyer was contacted with the soil and CO_2 output was provided. The volume of CO_2 emitted was recorded in the calcimeter. The amount of lime in % was found by calculating the obtained values [25].

2.4. Soil Humidity Analysis
10 g of agricultural soil samples taken for moisture analysis were weighed and left to dry at 105 °C. The dried soil samples were weighed again, the weight was noted and the % moisture content was calculated and determined.

2.5. Soil Nitrogen Analysis
Nitrogen amount in agricultural soil samples was determined by Semi-Micro Kjeldahl method [26]. For the method; Soil samples were weighed in a precision balance of 1 g. The weighed soil samples were placed in nitrogen combustion tubes and 2 pieces of Cu_2SO_4 + K_2SO_4 tablets were placed on them as catalysts. Afterwards, 12 ml of concentrated H_2SO_4 was placed in each tube and placed in the burning apparatus. Four of the tubes were prepared for blanking without a sample. Burning process was carried out for 1 hour at 420 °C. After waiting 10-15 minutes for the tubes to cool, blanking was performed with the samples in the Kjeldahl device and this value was entered into the Kjeldahl device by taking the average of the values. After entering each weighing value for the samples, the readings were made and the results were recorded and the calculation process was carried out.

2.6. Soil Enzyme Activities
For measurement of the dehydrogenase enzyme activity of the agricultural soil samples, analysis was applied according to the modified protocol of Tabatabai, 1994. Agricultural soil samples were weighed as 5 g according to the protocol and were treated with 5 ml 0.5% triphenyl tetrazolium chloride (TTC) and incubated at 37 °C for 24 hours. When the incubation period was completed, the samples were extracted with methanol, the resulting intensity of red color measurements were made at 480 nm wavelength in UV spectrophotometer and enzyme activity was calculated. The analysis was done in triplicate and the results were averaged.

In order to determine the phosphatase enzyme activities of the agricultural soil samples, 1 g of each sample was weighed and 0.25 ml of toluene, 4 ml of acetate buffer (pH 5.8) and 250µl of p-nitrophenol phosphate were added and left for 1 hour incubation at 37 °C. When the incubation period was completed, 1 ml 0,5 M $CaCl_2$ and 4 ml sodium hydroxide (0.5 M) was added and the measurement was made at 410

JSR A
Journal of Scientific Reports

nm wavelength in UV spectrophotometer. The enzyme activity was calculated and expressed as µg p-nitrophenol [27]. The results were averaged by performing three repetitions from each sample.

For determination of urease enzyme activity method of Tabatabai, 1994 [27] was used. 5 g of soil sample was weighed and it was treated with 0.2 ml of toluene, 9 ml of THAM (6.1 g Tris 0,2 M H_2SO_4) buffer solution and 1 ml of 0.2 mol /L urea solution. and left to incubation at 37 °C for 2 hours. When the incubation period was completed, enzyme activity was stopped by adding 35 ml KCl-Ag_2SO_4 solution. For the determination of NH4 + -N concentration, samples were extracted with 2 mol/L KCl and the ammonium concentrations in the extracts were measured using the indophenol blue method [28].

2.7. Mesophilic Bacterial Density

For calculating the number of total mesophilic bacteria, agricultural soil samples were prepared in various dilutions and inoculated on PCA (plate count agar) medium with the spread plate method. It was incubated for 96 hours at 37 °C. The total mesophilic bacteria number was calculated by counting the colonies formed as a result of incubation.

2.8. Biolog EcoPlate Test

Biolog EcoPlate (Biolog Inc., Hayward, California) was applied to determine the metabolic fingerprint of the microbial community in the agricultural soil samples. 10 g of soil samples were weighed and suspended in 90 ml of physiological saline water. It was shaked at 150 rpm and 37 °C for 1 hour. Then, the soil particles were left to settle and serial dilution was made with physiological saline water without touching the collapsed part. 150 µl of the 10^{-1} and 10^{-3} dilutions was added to the ecoplate wells. 150 µl of physiological saline water was added to the control wells. At the end of this process, the Biolog EcoPlates were left to incubate at 37 °C. Absorbances at 590 nm for up to five days at 24 hour intervals were measured at Biolog Microstation [29].

2.9. Statistics

In Biolog EcoPlate normalization process was applied to the each of the carbon source OD (Optical Density) values by using the readings in control wells OD values [30]. After normalization step, other analysis values were calculated from to data. One of the common analysis is kinetic analysis, which is time-dependent and a kind of color change analysis. At the end of the analysis as a result, a sigmoidal curve is often obtained. The AWCD values for all samples in each reading interval were calculated [31]. For analysis and calculations, the following formula (Eq.1) was used [32]:

$$\boldsymbol{AWCD} = \sum_{\boldsymbol{n=1}}^{\boldsymbol{31}} \left(\frac{\boldsymbol{ODi}}{\boldsymbol{31}}\right) \quad (1)$$

In the formula ODi means the normalized optical density of each well.
For calculation of AUC (Area Under the Curve) value, following formula (Eq. 2) was used [33]:

$$\boldsymbol{AUC} = \sum_{\boldsymbol{n=1}}^{\boldsymbol{31}} \frac{\boldsymbol{A_n + A_{n+1}}}{\boldsymbol{2x(t_{n+1} - t)}} \quad (2)$$

A_n and A_{n+1} is the absorbance value of each well in two consecutive time intervals. The t_n and t_{n+1} refers to two consecutive times.

JSR A
Journal of Scientific Reports

Incubation time effects and different parameters on the functional diversity were evaluated with calculations such as AUC and AWCD.
Data was obtained for 96 hours and then was used for calculations and analyses of diversity indices.
Shannon-Wiener functional diversity index (H) was calculated by the following formula (Eq.3), [34]:

$$\boldsymbol{H = -\sum_{n=1}^{31}(pi(Inpi)} \quad (3)$$

pi in the formula is the ratio of the absorbance of each substrate (ODi) to sum of the absorbance for the all substrates (∑ODi).

Substrate/catabolic richness (S) value was calculated for every 24 hours as the total number of oxidized substrate (C). The total number of the oxidized substrates were determined which were wells in the absorbance value over 0.25.

In addition to other calculations, Shannon Evenness (E) index was determined by the following formula using these values (Eq.4):

$$\boldsymbol{E = \frac{H}{InS}} \quad (4)$$

3. RESULTS AND DISCUSSION

In this study four different agricultural soils were evaluated. For a sustainable production system, soil must have high and stable biological diversity and nutrient cycling [35]. Living and non-living factors in the ecosystem cause changes in the microbial community structure. Some of these factors are humidity, type of plant, nutrient concentrations and temperature [36]. The microbial cells have some adaptations to survive in different environmental conditions [37]. In the light of these explanations, both the physicochemical properties of the soil were determined and the enzymatic and metabolic potentials were measured within the scope of microbial fertility.
The pH measurement and electrical conductivity results of the agricultural soil samples were shown in Table 1. Average pH measurements of samples were recorded around 8.2. Their electrical conductivity varied between 406-524.5 µS cm^{-1}.

Table 1. pH and EC values of agricultural soil samples

Soil Samples	pH (-log[H+])		Electrical conductivity (EC) (µS cm-1)
A	8.22	Medium alkaline	447.5
B	8.27	Medium alkaline	429
C	8.23	Medium alkaline	524.5
D	8.21	Medium alkaline	406

Information on calcium carbonate values, humidity rates anad nitrogen amounts determined in agricultural soil samples were given in Table 2. When the data was examined, soil samples were

defined as calcareous and very calcareous. The % moisture content of soil samples were calculated average nearly 30%. The amount of nitrogen determined by the Semi-Micro Kieldahl method in soil samples were varied between 0.1 and 0.3.

Table 2. % lime values of agricultural soil samples.

Soil Samples	Lime (%)		Humidity rate (%)	Nitrogen Content (%)
A	17.576	Very calcareous	30.718	0.3304
B	17.210	Very calcareous	38.312	0.1876
C	13.365	Calcerous	41.043	0.1484
D	11.534	Calcerous	37.551	0.3723

For the bacteria count, the number of aerobic mesophilic bacteria was calculated by counting the colonies formed after 96 hours of incubation at 37 °C. The calculation results were given in Table 3. Although there was a significant difference between the calcium carbonate content of soil samples, there was no significant difference of soils' pH and EC. These differences may be due to rotation, tillage, residue type and fertilization time on different agricultural soil samples. Measurements and controlling pH, EC and moisture values were crucial for agricultural soils biological balance and and associated with plant productivity [38, 39].

Table 3. Total mesophilic bacteria count of agricultural soil samples.

Soil Samples	Number of mesophilic bacteria (cfu/ml)
A	2.66×10^6
B	1.2×10^6
C	2.02×10^6
D	4.365×10^6

Dehydrogenase enzyme activity, phosphotase enzyme activity and urease enzyme activity of soil samples were given in Table 4. Soil microbial communities change when exposed to different agricultural practices. Soil enzyme activity is correlated with indirectly soil microbial activity [40]. One of these enzymes is urease. This enzyme hydrolyzes the urea fertilizers used in agricultural applications. However urease can cause to increasing in pH [41]. Urease activity values were nearly same, but in A sample was in low level than the others. The activity of dehydrogenases and phosphatase enzymes is directly related to the presence of carbon, nitrogen and phosphorus for plants [42]. If the organic carbon level in the soil is high, microbial activity and enzyme activity are also high. Dehydrogenase enzyme is an indicator of viable microbial cells, microbial respiration and microbial activity in samples [43]. Alkaline phosphatase is another enzyme that is responsible for hydrolization of phosphours [44]. Enzyme activity results show correlation with organic carbon. Soil enzymes are found to be significantly compiled into soil health. In our study dehydrogenease activity and cell count results were in positive correlation.

JSR A
Journal of Scientific Reports

Table 4. Enzyme activity results of agricultural samples.

Soil Samples	Dehydrogenase enzyme activity (µg TPF g^{-1} soil/ h^{-1})	SD(±)	Phosphotase enzyme activity (µg p-nitrophenol)	SD(±)	Urease enzyme activity (µg NH_4-N g^{-1} soil h^{-1})	SD(±)
A	39.219	7.929	8.876	0.472	94.479	1.183
B	30.592	2.020	11.422	3.592	118.500	1.621
C	38.110	4.517	9.950	0.807	110.708	1.270
D	33.628	1.92	11.617	3.583	132.437	1.738

Similar to other results, positive correlation was obtained between soil biodiversity indicators and the main groups of compounds in the Biolog EcoPlate analysis. Also, the soils collected from different agricultural region revealed the highest biological activity and diversity. AWCD, Shannon-Wiener functional diversity index (H), catabolic richness (S), Shannon Evenness (E) index and AUC (Area Under the Curve) values of agricultural soil samples were shown in Table 5.

Table 5. AWCD, (H), (S), (E) and AUC values of agricultural soil samples.

Samples (24 hour)	Indices				
	AWCD	H	E	S	AUC
A	0.703±0.053	3.424 ± 0.005	0.997 ± 0.001	31	
B	0.749± 0.010	3.425 ± 0.008	0.997 ± 0.002	30.667 ± 0.577	
C	0.756 ± 0.014	3.429 ± 0.003	0.999 ± 0.001	31	
D	0.796± 0.023	3.426 ± 0.005	0.998 ± 0.001	31	
Samples (48 hour)	**Indices**				
	AWCD	H	E	S	AUC
A	0.727± 0.057	3.424± 0.003	0.997 ± 0.001	31	
B	0.802± 0.048	3.424± 0.010	0.997 ± 0.003	30.667 ± 0.577	
C	0.978 ± 0.069	3.401± 0.012	0.990 ± 0.004	31	
D	0.900± 0.061	3.420± 0.010	0.996± 0.003	31	
Samples (72 hour)	**Indices**				
	AWCD	H	E	S	AUC
A	0.869± 0.148	3.407± 0.011	0.992 ± 0.003	31	
B	0.926± 0.115	3.405± 0.025	0.991 ± 0.007	30.667 ± 0.577	
C	1.189 ± 0.123	3.388± 0.005	0.987 ± 0.001	31	
D	1.029± 0.149	3.408± 0.021	0.992± 0.006	31	
Samples (96 hour)	**Indices**				
	AWCD	H	E	S	AUC
A	1.062± 0.243	3.374± 0.007	0.983 ± 0.002	31	59.484
B	1.028± 0.115	3.389± 0.026	0.987 ± 0.007	30.667 ± 0.577	62.789
C	1.320 ± 0.093	3.366± 0.004	0.980 ± 0.001	31	76.938
D	1.040± 0.167	3.400± 0.031	0.990± 0.009	31	68.321

Substrates were grouped as carbohydrates, carboxylic acids, amino acids, polymers, and amines/amides for comparision. In most of the soils, all of the carbon sources were used. This was resulted by the high diversity index values. Figure 2 and 3 shows carbon utilization heat map after 24 and 96 hours.

JSR A
Journal of Scientific Reports

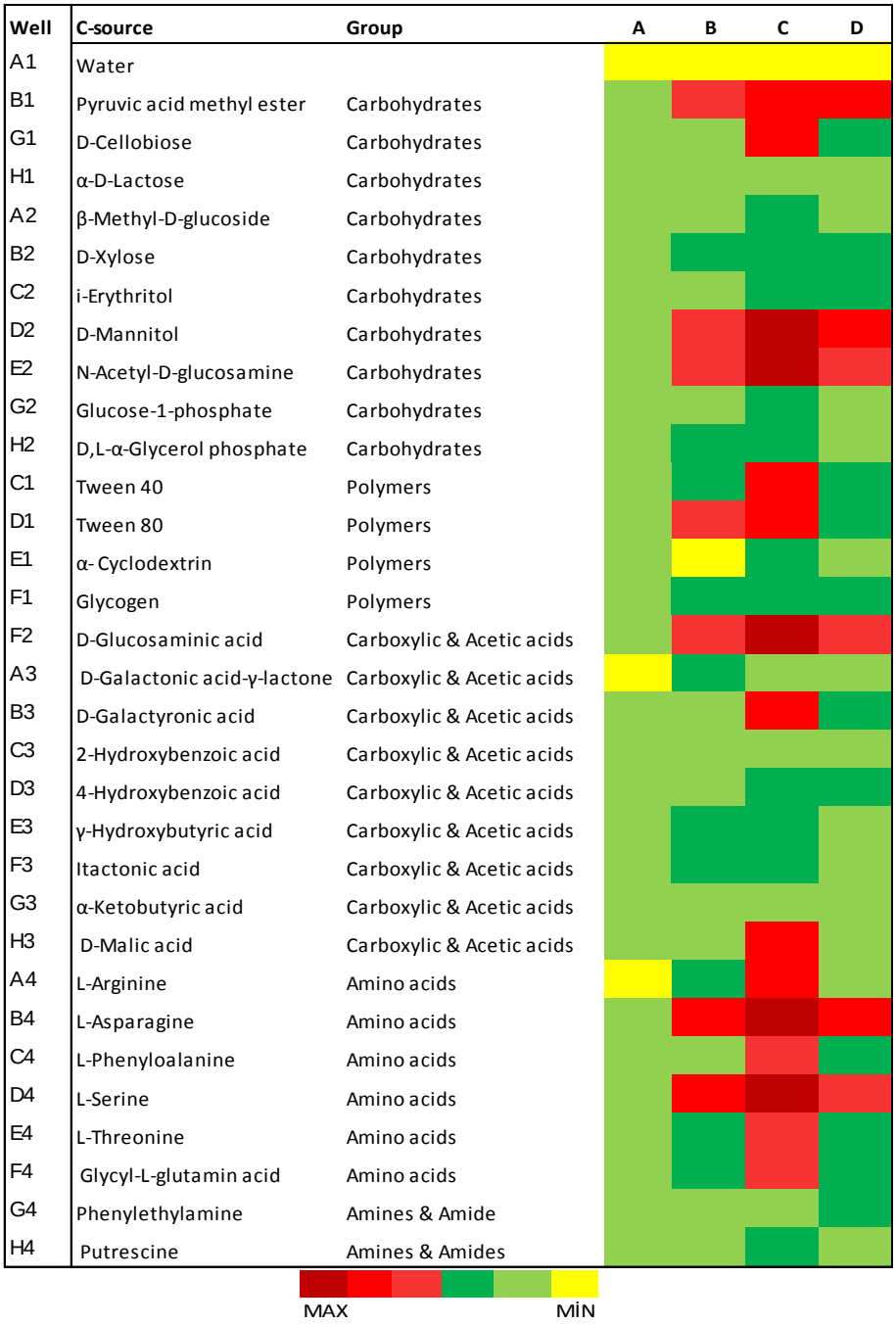

Figure 2. Carbon utilization heat map after 24 hours.

JSR A
Journal of Scientific Reports

Well	C-source	Group	A	B	C	D
A1	Water					
B1	Pyruvic acid methyl ester	Carbohydrates				
G1	D-Cellobiose	Carbohydrates				
H1	α-D-Lactose	Carbohydrates				
A2	β-Methyl-D-glucoside	Carbohydrates				
B2	D-Xylose	Carbohydrates				
C2	i-Erythritol	Carbohydrates				
D2	D-Mannitol	Carbohydrates				
E2	N-Acetyl-D-glucosamine	Carbohydrates				
G2	Glucose-1-phosphate	Carbohydrates				
H2	D,L-α-Glycerol phosphate	Carbohydrates				
C1	Tween 40	Polymers				
D1	Tween 80	Polymers				
E1	α- Cyclodextrin	Polymers				
F1	Glycogen	Polymers				
F2	D-Glucosaminic acid	Carboxylic & Acetic acids				
A3	D-Galactonic acid-γ-lactone	Carboxylic & Acetic acids				
B3	D-Galactyronic acid	Carboxylic & Acetic acids				
C3	2-Hydroxybenzoic acid	Carboxylic & Acetic acids				
D3	4-Hydroxybenzoic acid	Carboxylic & Acetic acids				
E3	γ-Hydroxybutyric acid	Carboxylic & Acetic acids				
F3	Itactonic acid	Carboxylic & Acetic acids				
G3	α-Ketobutyric acid	Carboxylic & Acetic acids				
H3	D-Malic acid	Carboxylic & Acetic acids				
A4	L-Arginine	Amino acids				
B4	L-Asparagine	Amino acids				
C4	L-Phenyloalanine	Amino acids				
D4	L-Serine	Amino acids				
E4	L-Threonine	Amino acids				
F4	Glycyl-L-glutamin acid	Amino acids				
G4	Phenylethylamine	Amines & Amide				
H4	Putrescine	Amines & Amides				

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Figure 3. Carbon utilization heat map after 96 hours.

JSR A
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Compared to other samples, C and D agricultural soil samples had a higher overall AWCD value. The microbial communities in the C and D soil samples utilized more amino acids than the others. The differences in the use of substrate as a result of the analysis of different soils indicate the various metabolic capabilities of soil microbial communities. There can be many reasons for these results, for example, nitrogenous fertilizer application changes and affects the catabolization ability of soil microorganisms. AWCD values are related with the oxidative ability of microorganisms. The same results were obtained for AUC values. The metabolically richest samples were C and D soil samples. High AWCD, H, R, S, E and AUC results show microbial activity and high number of oxidized C substrates [45, 46]. In Biolog EcoPlate results after 96 hours incubation, it was detected that 31 carbon sources used. However, the presence of carbon sources with a lower utilization rate was detected within 24, 48 and 72 hours. Carbon utilization profiles were different of the soil samples as shown in Figure 2 and 3. Evaluation of samples carbon source utilization potentials, some sources were utilized slowly within the first 24 hours. In B soil sample α- Cyclodextrin, α-Ketobutyric acid, β-Methyl-D-glucoside, α-D-Lactose were used slowly. In C soil sample, all sources were consumed efficiently within the first 24 hours especially amino acid and carbohydrate groups. These results showed in C soil sample have to the high degradation and catabolic capacity also may have high number of heterotrophic bacteria. AWCD calculation results indicated that C and D soil samples had highest values. AWCD values can be showed the lag and exponential phases of microorganisms. Lag phase can be show that the microorganism number is low. In this phase microbial community adapt to substrate degradation and synthesize new enzymes. A and B soil samples reached exponential phase too late than C and D as shown in Figure 4.

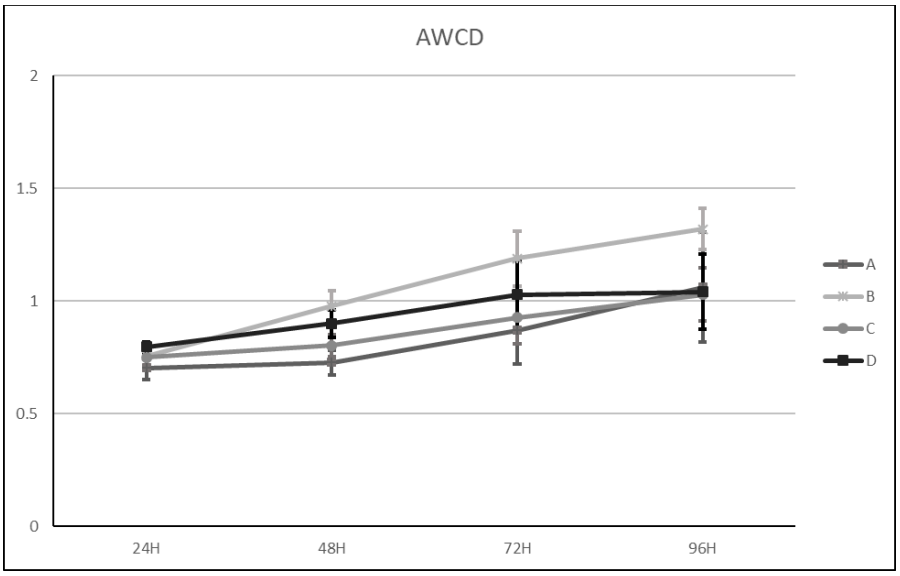

Figure 4. AWCD values for agricultural soil samples.

Biolog EcoPlates is a common method to characterize changes in microbial community of soils [17, 47]. This technique supplies a quick results about community structure. In this study experimental

findings revealed that substrates utilization profiles and the high diversity indices values indicated highly active and diverse microbial communities.

4. CONCLUSIONS

Our results showed that enzymatic values and Biolog EcoPlate are potential alternative tools to detect soil microbial communities' structure and changes, physiological properties and utilized carbon sources. These methods are practice and economical. In our preliminary study we used enzymatic methods for bacteria monitoring. These preliminary results can be used for efficient agriculture treatments. If necessary alternative methods and detailed analysis may be apply for detailed community structure analysis.

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