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Biodegradation of glyphosate herbicide by *Salinicoccus* spp isolated from Qom Hoze-soltan lake, Iran

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Abstract

Background: Glyphosate (N-phosphonomethyl Glycine) is an organophosphorus pesticide with dangerous effects on the environment. In this study, the biodegradation of glyphosate herbicide by halophilic bacteria isolated from Qom Hoze-Soltan Lake has been investigated.

Methods: After sampling and bacterial isolation, native halophilic strains grown in the presence of glyphosate at a wavelength of 660 nm and also the disappearance of the glyphosate in the plates at a wavelength of 220 nm were determined and the dominant bacteria were isolated. Biochemical, molecular (according to the 16S rRNA sequence), antibiotic, and the Minimum Inhibitory Concentration (MIC) test was performed for the dominant bacteria. Analysis of the remaining glyphosate herbicide was performed by HPLC analysis after derivation with FMOC-Cl.

Results: According to the results of the biochemical, antibiotic and molecular 16S rRNA tests, the native halophilic isolates with the ability to biodegrade glyphosate were gram positive cocci very similar to *Salinicoccus* spp. The results of HPLC showed that *Salinicoccus* spp is able to biodegrade glyphosate herbicide.

Conclusion: The native bacteria in Qom Hoze-soltan lake, Iran can be used for biodegradation of glyphosate herbicide.

Keywords: Glyphosate, Biodegradation, Salinicoccus spp

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Introduction

The increase in human population and therefore increase in the demand for agricultural products has led humans to search for more efficient methods for increasing agricultural products. Weed control is one of these methods. Nowadays, humans control weeds by mechanical or chemical methods. One of the chemical methods is using herbicides. The sales of herbicides in 2006 were about 3 billion liters. One of these herbicides is glyphosate (N-phosphonomethyl Glycine), commercially known as roundup. Glyphosate broad-spectrum and nonselective systemic herbicide, is described by the possession of a stable and covalent Carbon-to-Phosphorus (COP) bond. Glyphosate is utilized extensively for control of plants including grass, sedge, broad-leaved weeds and woody plants. It can be used on non-cropland as well as on a great variety of crops. Demand for glyphosate has increased substantially in the world. Ho_4 has been reported a 15-fold increase in usage of glyphosate on major crops between 1994 and 2005. Glyphosate itself is an acid, but it is commonly used in salt form, most commonly isopropyl-amine salt (1,2). This herbicide is from the phosphoric acid group, and blocks the EPSPS enzyme by disturbing the plant's synthesis of amino acids and enzyme activity, and blocks its shikimate pathways (1). Considering the fact that most of the agricultural soil in Iran is saline soil, the environmental problems derived from harmful chemicals especially organophosphorus chemicals in saline environments have been known for long, but our information about the contaminants of these environments is still little, because the halophilic microorganisms that degrade these toxic chemicals have not yet been evaluated. Due to the wide range of used pesticides, it is difficult to produce a single method for the removal of pesticide from water and wastewater. Thus, there are several treatment processes for the removal of pesticides – e.g. biodegradation, photodegradation, oxidation (with air, chlorine, permanganate or ozone), flocculation and filtration, adsorption

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and membrane techniques. As a general rule, biological treatment is more economical than physicochemical remediation methods as it can be cost-effective and achieve the complete degradation of organic pollutants (3).

Microorganisms that degrade glyphosate act in two ways. One path leads to the intermediate formation of sarcosine and glycine, and the other path leads to the formation of AMPA or 2-amino-3(5-methyl-3-oxo-1,2-oxazol-4-yl) propanoic acid (4,5). Halophilic microorganisms, are the ones that are capable of living in environments with high and low temperatures, with acidic and alkaline pH, high hydrostatic pressure, and high salt concentrations (6). In Iran there are many lakes and saline ecosystems in which the concentration of sodium and chloride ions are high. One of these salinas is the Hoze-Soltan lake in Qom. The salt march and arable soils around this lake make it one of the most important and rich sources of halophilic bacteria (7).

The aim of this study was to evaluate the degradation glyphosate herbicide by halophilic bacteria from the environmental soil samples collected from 5 different districts around the lake.

Methods

Isolating halophilic bacteria

The medium for isolating halophilic bacteria includes; 51 g/l NaCl, 0.026 g/l NaBr, 15 g/l Agar-agar, 9.7 g/l $MgSO_4.7H_2O$, 5 g/l Yeast-extract, 5 g/l Polypeptone, 2 g/l KCl, 0.06 g/l $NaHCO_3$, 10 g/l Glucose, 7 g/l $MgCl_2.6H_2O$, 3.6 g/l $CaCl_2$, and 1000 ml distill water (3).

In order to isolate the halophilic microorganisms, Broth and Agar medium were used. The concentration of salt in the environment was between 5% to 10% and the pH of the water; saline soil, and salt sedimentsrange between 6.5 and 8.2. Major chemical composition of the soil, brine, mud, and salt consists of NaCl, KCl, $MgSO_4$, $MgCl_2$, and Na_2SO_4 and the amount of herbicide used is 500 ppm which is equal to the that of this herbicide used in soil (3,8). The important fact is that instead of glucose, the glyphosate herbicide was used in this medium, in other words glyphosate was used as the source of carbon in this medium. This herbicide was added after autoclaving and filtering to the medium. About 1 gram of soil in 250 ml Erlenmeyer flask containing 100 ml of culture medium and 1% glyphosate was added and incubated at 34°C shaking at 150 rpm for 7 days. Then it was inoculated and incubated in the agar medium at 30°C for 48 hours. After incubation (in 30°C for 48 hours), the bacteria were detected with diagnostic tests. Eventually the isolating and purifying processes were done.

Diagnosing halophilic bacteria

After gram staining the bacteria and spores staining, the initial identification and biochemical tests including motivation tests, oxidation tests, catalase tests, gelatin-starch-caseine hydrolysis tests, Tween 80 hydrolysis tests, Bile Esculin hydrolysis tests, Nitrate reduction test, H2S production, acid production from sugar (glucose, lactose, raffinose, arabinose, mannitol) and tolerance and sensitivity to antibiotic tests including chloramphenicol, penicillin G, tetracycline, nalidixic acid, cephalothin, ampicilin, rifampin, erythromycin, and streptomycin were performed (9).

Determining herbicide MIC

In order to determine the sensitivity of the toxin and bacteria, initially a thick solution (4500 ppm) of the glyphosate herbicide was made. Then in sterile tubes containing the Mueller Hinton Broth medium with 10% salt, 1.25 ml of herbicide was added and the solution was prepared. Then 0.01 ml of the bacteria under study equal to 0.5 according standard microbial test was added to the solutions and was placed in an incubator in 30°C. After 48 hours the samples were checked for turbidity and the MIC was determined (10).

Evaluating the logarithmic growth of bacteria in the presence of herbicide

In order to evaluate the 10 isolated bacteria's degradation ability, initially specific sterile mediums including NaCl 10% were made and the herbicide was added and then bacteria equal to 0.5 McFarland was injected. One flask was used as blank for the spectrophotometer and another flask with salt and herbicide was used as control.

The flasks were placed in a shaker incubator in 30°C and 150 rpm, and after 48 hours, their absorption was measured by the spectrophotometer in 660 nm wave lengths for evaluating growth and in 220 nm for evaluating degradation of the herbicide (9,10). The best isolates with maximum absorption at 660 nm and minimum absorption at 220 nm were chosen.

In order to choose the best isolates based on optimum growth in the presence of herbicide, their growth during different hours of incubation in wave lengths of 660 nm were measured (Figure 1).

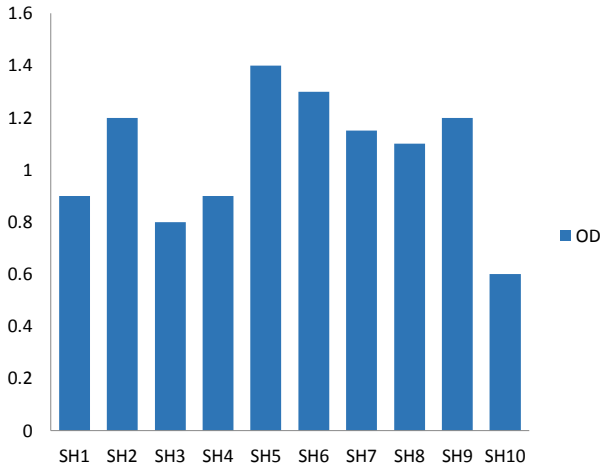

Figure 1. Evaluating the growth capability of halophilic isolates in the presence of glyphosate herbicide.

Analysis of glyphosate degradation by Thin Layer Chromatography (TLC)

In order to do this experiment, cellulose chromatography paper and solutions of ethanol, water, ammonium hydroxide (17 molar), trichloroacetic acid and acetic acid (15 molar) with proportions of (2:3.5:2.5:35:55 v/w/v/v/v) were used (11,12).

After 48 hours the best isolated grown bacteria were taken from the top of the solution in the flask and were placed on chromatography paper. Also glyphosate herbicide was used as control and was placed on chromatography paper. Then, the paper was placed in a dish including a prepared solvent. After a while the paper was viewed by UV light in 220 nanometers (6).

Analysis of glyphosate herbicide by HPLC

The material needed for HPLC were glyphosate, acetonitrile (HPLC grade), potassium dihydrogen phosphate, disodium tetraborate decahydrate, HCL 32%, KOH 4 M, FMOC-CL (9-Fluorenylmethyl)-chloroformate, ethyl acetate (HPLC grade), the HPLC device, and ultra pure water (9,13).

The preparation method for injecting the samples to HPLC

After 48 hours, the best grown isolates in the shaker incubator were taken from the top of the solution in the flask and were centrifuged for 10 minutes at twice in 12,000 rpm. Then the top solution was filtered through a Millipore filter. After making sure that the solution was clear, it was concentrated by the rotary device in 40°C. Also one sample was used as control (9).

For preparing the 1.5% phosphate buffer, the amount of 7.5 grams of KH_2PO_4 was dissolved in 500 ml of ultra pure water. The borate buffer 0.05 M was made by dissolving 1.9 grams of disodium tetraborate decahydrate in 100 ml of ultra pure water. 10 mg of FMOC-Cl was dissolved in 10 mm of acetonitrile in order to prepare 1000 ppm FMOC-Cl (9).

The pre-column derivation method before injecting into the HPLC

The amount of 3 ml of the specified sample (clear and concentrated) with 0.5 ml of 0.05 M Borate Buffer and 0.5 ml of 1000 ppm FMOC-Cl solution was poured in a vial and was placed for one hour in the room temperature. Then in order to eliminate the extra derivative, it was washed by 2 ml ethyl acetate and the aqueous phase was injected into the HPLC (9).

Calibrating the HPLC

The column used in this device was a C18 (4.6 mm×250 mm) column (14). The mobile phase used included acetonitrile and 1.5% phosphate buffer with pH=5.8 proportionate to 45/55. The type of detector used was florescent. The flow rate was 1 ml/min, and the amount of sample injected was 50 µL, ex λ was at 260 nm and em λ was 310 nm. Then the sample was injected into the HPLC (9,15).

The DNA extraction process from the bacteria was performed by the Bioneer Extraction Kit. In order to confirm the extraction of DNA, electrophoresis was used. Then in order to do PCR these listed primers were used (7).

8F: AGAGTTTGATMLTGGCTCAG

1541R: AAGGAGGTGATMLAGMLGCA

The PCR reaction started with the initial melting temperature of 95°C for 5 minutes and continued with 10 cycles in 93°C for 1 minute, 63°C for 1minute, 72°C for 1 minute, and finished with 20 cycles in 93°C for 1 minute, 67°C for 1 minute, 72°C for 1 minute, and eventually 72°C for 5 minutes (6).

Results

Results from bacteria logarithmic growth and toxin degradation

Diagnostic tests revealed that bacteria are gram-positive, catalase-positive, oxidase-positive, non-sporeforming bacteria, and non-motile and has the ability to grow in salt, 1% to 15%. The SH5, SH6, and SH9 isolates from the 10 halophilic native bacteria showed good activity in degrading glyphosate herbicide which is valuable, and the SH5 isolate had the highest growth rate among the isolates taken from region 5 (soil beside plants).

As it can been seen in Figure 2 the SH5, SH6, and SH9 isolates have shown a better activity in comparison to the other isolates and decreased the amount of herbicide. The SH5 isolate in comparison to the other isolates decreased the herbicide more and had better capability in degrading glyphosate herbicide.

Eventually 3 isolates from the 10 isolates, had a higher activity in degrading the glyphosate herbicide in comparison to the other isolates. In order to choose the best isolates, their growth in different hours during incubation was evaluated.

The SH5 isolate had a better function in regard to growth and degrading the glyphosate herbicide. Figure 3 shows that the isolates taken from soil in the presence of this herbicide in the first 20 hours after the inoculation of bacteria are in lag phase and show little growth. After 36 to 48 hours, they grow abruptly and fast, and this accelerated growth continues with less speed to 120 hours later. Eventually their increased growth stops and bacteria enter a stationary phase.

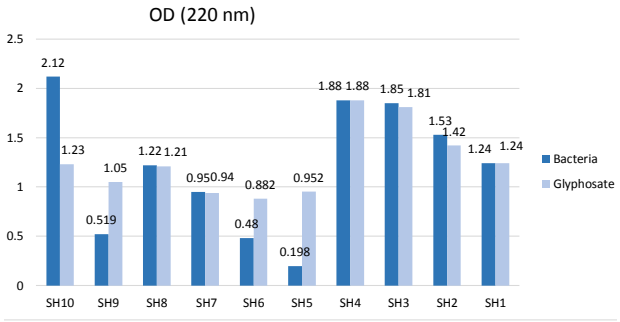

Figure 2. Evaluating the capability of halophilic isolates in degrading the glyphosate herbicide in comparison to control

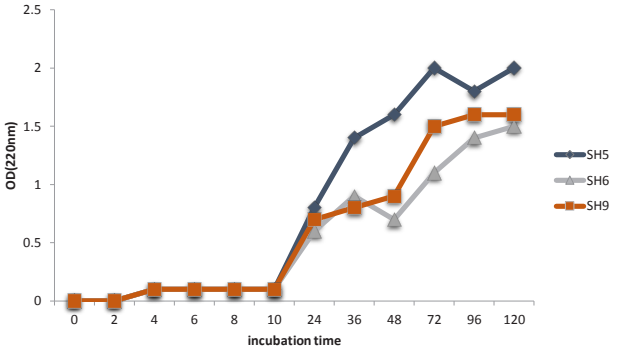

Figure 3. Comparing the growth of 3 dominant halophilic isolates in degrading the glyphosate herbicide.

Determining the MIC of the dominant SH5 bacteria

After experimenting, we found out that the SH5 isolate can tolerate the glyphosate herbicide up to 2250 ppm after 48 hours incubation.

Thin layer chromatography

The SH5 isolate is capable of degrading the glyphosate herbicide after 48 hours incubation. In the picture below, the result of the experiment can be seen. The right column (column 2) is the control (glyphosate herbicide), and its band can be seen. The left column (column 1) is the 48-hour result of the SH5 isolate, and no band can be seen (Figure 4).

The HPLC analysis

As it can be seen in Figure 5, initially a big peak that lasts between 3 to 4 minutes and is related to FMOC-OH was seen. Then, other peaks were seen at 7 and 9.5 minutes. And eventually the peak related to glyphosate herbicide was seen at 16.567 minutes.

In Figure 6, initially a big peak between 3 to 4 minutes can be seen which is related to FMOC-OH. Then another peak at 6.247 minutes can be seen which is related to

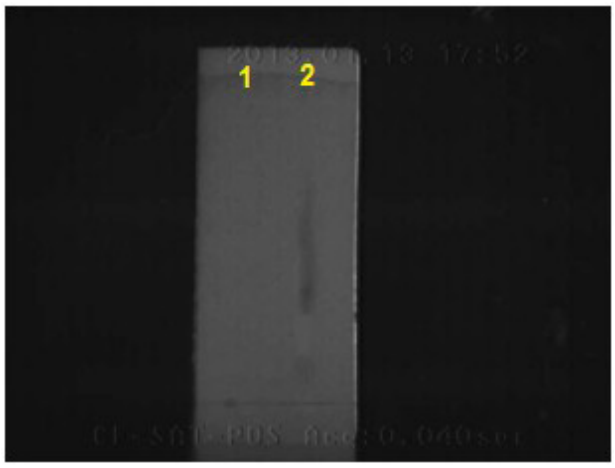

Figure 4. The chromatography result of TLC from the 48-hour sample of the SH5 isolate and glyphosate herbicide

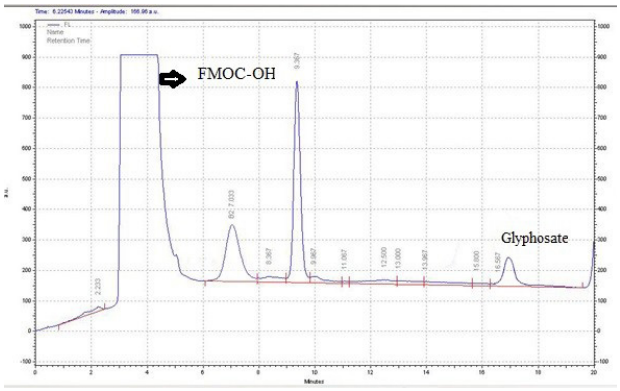

Figure 5. The chromatography of glyphosate herbicide from HPLC after derivation by FMOC-Cl.

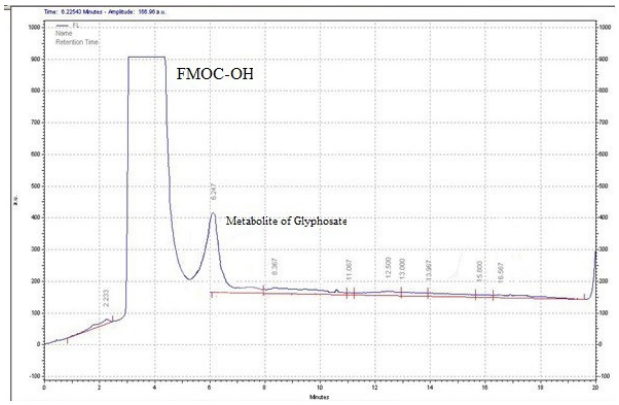

Figure 6. The chromatography of treated glyphosate herbicide with the dominant isolated by HPLC after derivation by FMOC-CL

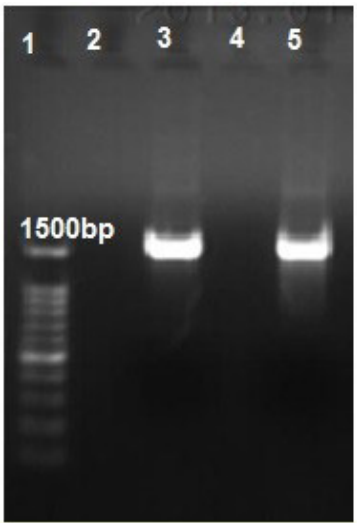

Figure 7. The electrophoresis gel from PCR in the gel displaying device (Column 1: marker, Column 2 space, Column 3: the specified sample which includes 2 microliters of DNA, Column 4 : space, Column 5 :the specified sample that includes 3 microliters of DNA)

the metabolite extracted from the 48-hour concentrated and derived sample (SH5 isolate), and as it can be seen at 16.567 minutes, the peak related to glyphosate herbicide cannot be seen.

The PCR experiment of 16S rRNA gene

The band at 1500 bp in the PCR reaction shows the proliferation of 16SrRNA piece.

Molecular analysis

The band from proliferation of the 16S rRNA gene in PCR was sent to Iran Pasteur Institute for determination. Evaluation and comparison of the sequence of 16S rRNA from bacteria isolated from the salty soil of Hoze-Soltan lake, with the available sequences at the NBCI information bank showed that the bacteria isolated was strain QW6 *Salinicoccus* sp. (DQ767692) (Figure 7).

Discussion

Herbicides are chemicals that are used for weed control. When herbicides are added to soil, the consequences of their presence might be critical. Their long presence in soil can lead to elimination of the beneficial microorganism in soil. Therefore their biodegradation is important and is the best alternative for preventing their accumulation and environmental stability. Now, we can achieve this goal by isolating bacteria that can degrade this herbicide. Usually microbial ways for eliminating glyphosates are more effective than chemical ways, because herbicides have a strong carbon-phosphorus bond that can be degraded by these bacteria (11).

According to the result of these experiments, the dominant halophilic bacteria can be effective in degrading this herbicide up to 2250 ppm, but higher concentrations of the herbicide have a prohibiting effect, and the best growth is seen in 1080 ppm.

Monke *et al* in 2010 evaluated the effect of different concentrations of the glyphosate herbicide in non-saline environments and in regular conditions in the *Acetobacter* sp and *P. fluorescens* bacteria and found out that the best growth was in concentrations equal to 7200 ppm, and these two bacteria could tolerate herbicides up to 10,000 ppm (16).

Ermakova *et al* in 2010 claimed that none of the two *Achromobacter* sp and *Ochrobactrum anthropi* bacteria could use glyphosate as a source of carbon and phosphorus. Both bacteria grew in an environment that the herbicide and methyl phosphonic were added as a source of phosphorus; and were able to break the C-P bond in glyphosate (4).

Researchers have been able to eliminate the dichlorvos herbicide from saline environments by halophilic bacteria. Four halophilic isolates from the Techirghiol lake in Romania were able to affect glyphosates.

Researcher observed that the semi-halophilic bacteria such as *Chromohalobacter marismortui* can degrade methy-, ethyl-, 3-aminopropyl and 4-aminobutyl- phosphonate which is an organophosphorus compound. The optimum concentration of salt used in this environment was 10%, and they were able to grow in 2% to 25% concentration of salt and pH=7.2 (13).

Other bacteria that have been identified in degrading glyphosates are *Arthrobacter atrocyaneus* and *flavobacterium* sp (1,5). Researchers think that the bacteria in soil are the only organisms that degrade glyphosate (14). Also *Pseudomonas* sp isolate LBr (15) *Acetobacter atrocyaneus* (13), and *Flavobacterium* sp bacteria (1) were able to degrade the glyphosate herbicide. Adelowo *et al* discovered this first fungal degradation of glyphosate by penicillium citrinum (17).

The results from HPLC with fluorescent detector in this research after extraction, concentration, and derivation by FMOC-Cl showed a big peak which is specific to FMOC-OH at 3 minutes that lasted for 4 minutes and the peak for glyphosate herbicide was at 16.567 minutes and the peak for the metabolites of degradation was seen at 6.247 minutes.

In 2008, researchers able to obtain the detector peak of glyphosate herbicide at 10.013 minutes and AMPA (the main metabolite of the glyphosate herbicide) at 6.3 minutes after deriving the glyphosate herbicide and AMPA by FMOC-Cl and by the florescent HPLC (18).

Bot *et al* in 2002 observed one big peak at 3 minutes which lasted for 4 minutes and was related to FMOC- OH, a peak related to glyphosate herbicide at 16.344 minutes, and a peak related to AMPA (the main metabolite of the glyphosate herbicide) at 6.05 minutes after deriving the glyphosate herbicide and AMPA by FMOC-Cl and by the fluorescent detector HPLC (2).

Conclusion

Halophilic bacteria isolated from Qom Hoze Soltan lake can grow in the presence of herbicide and can metabolize this environmental contaminant.

Acknowledgments

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Ethical issues

We certify that all data collected during the study is presented in this manuscript and no data from the study has been or will be published separately.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

All authors were involved in study design, data collection, and article approval.

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