VNU Journal of Science: Natural Sciences and Technology, Vol. 32, No. 1S (2016) 353-361

Enrichment and Selection of Microbial Communities Capable of Degrading the Herbicidal Pollutant Glyphosate

Hoang Thi Oanh, Pham The Hai*

VNU University of Science, 334 Nguyen Trai, Hanoi, Vietnam

Received 15 July 2016
Revised 25 August 2016; Accepted 09 September 2016

Abstract: Weed control is an inevitable practice in agricultural systems. One of the most applied herbicides in the world was glyphosate. However, levels of glyphosate residues in Vietnam and over the world were alarmingly high. Currently, chemical methods and biological methods using single microbial strains are applied for the degradation of glyphosate treatment but still have some limitations. Therefore, this study aims at finding a microbial community capable of efficiently degrading glyphosate. Microorganisms from different samples were enriched by the dilution method on a selective medium containing glyphosate. The results showed that three microbial communities having the desired capability were successfully enriched, designated as SH, CP and LS. The solutions containing glyphosate and previously treated with an enriched community (SH) was proven to be nontoxic to plants. The SH enriched community appeared to have a more efficient glyphosate-degrading capability, compared to those of its single individual strains (*Sphingomonas* sp (SH1), *Ochrobactrum* sp (SH2), *Enterobacter cloacae* (SH3) and *Pseudomonas* sp (SH4)). A specific community composition and a synergistic community harmonization might be the reason for the better performance of the SH enriched community compared with its single individual strains as well as the other communities. This result indicates that research on the use of mixed cultures in bioremediation (instead of using single strains) is necessary and thus deserves more attentions in the future.

Keywords: Degradation, herbicide, glyphosate, microbial community, bioremediation, *Sphingomonas* sp., *Pseudomonas* sp.

1. Introduction

Glyphosate [*N*-(phosphonomethyl) glycine], a non-selective and lately-emerging herbicide, has been widely used to eliminate weeds. It was discovered to be a herbicide by the Mosanto chemist John E. Franz in 1970 and became one of the most popular herbicides in the world used in agriculture [1]. This herbicide kills plants by blocking the activity of 5-enolpyruvoyl-shikimate-3-phosphate synthetase (EPSPS) responsible for producing aromatic amino acids such as phenylalanine, tyrosine and tryptophan. Without these amino acids, a plant cannot synthesize proteins required for its life processes, and thus can be dead [2, 3, 4] Since 1995, the use of glyphosate has exponentially increased with the planting of genetically engineered glyphosate-tolerant crops.

*Tác giả liên hệ. ĐT.: 84-913318978
Email: phamthehai@vnu.edu.vn

According to the National Brazilian Environmental Council (CONAMA), the maximum concentration of glyphosate allowed in fresh water is 0.280 mg/L. However, Chang *et al.* revealed that glyphosate was detected in water, rain and air in the Mississippi River basin with high concentrations of up to 2.5 μg/L. Because glyphosate is dispersed in air, water and food, it is likely to be accumulating in the human body with low doses over time. Concentrations of residual glyphosate of up to 4.4 parts per million (ppm) have been detected in stems, leaves and beans of glyphosate-resistant soy.

In May 2012, according to Nha Trang Pasteur Institute in Vietnam, water samples containing about 1 mg/L glyphosate and particularly soil samples containing high levels of 14.3 mg/kg glyphosate were detected from Làng Riềng village, Sơn Ky Commune, Sơn Hà mountainous district, Quãng Ngãi province. This herbicide is considered as a "murderer" causing 3 deaths and over 50 cases of eye damages in April, 2012 [5].

A cautious alarm of glyphosate pollution in Vietnam rang again in August 2013. Local people in Quảng Bình province found out that herbicides including glyphosate were applied in Bố Trạch afforestation yards to eliminate unwanted plants on an area of 3 ha. Therefore, removal or degradation of residual glyphosate has become a very important topic not only in the world but also in Vietnam.

For the treatment of glyphosate, some chemical methods in laboratory were used, such as photodegradation of glyphosate in a system using ferrioxalate and the oxidative degradation of glyphosate on manganese oxide [6, 7, 8]. However, these methods are rather complicated and time consuming. In addition, the efficiency of photodegradation of glyphosate is not really high, at around 60% [6].

In recent years, biodegradation methods, particularly the use of bacterial single strains, have been more widely studied and popularly applied [9, 10]. Olawale and colleagues have indicated that strains of *Pseudomonas putida*, *Pseudomonas aeruginosa* and *Acetobacter faecalis* isolated from agricultural soil heavily polluted with glyphosate (at 1000 ppm) are capable of degrading this substance [11]. Nevertheless, the use of single strains may face some challenges relating to their adaptation to the natural environment and competition for resources with indigenous populations. Furthermore, the single strains may not have the optimal degradation efficiency in the natural environment because the conditions in the environment are far more complex than the laboratory conditions. Hence, the use of microbial communities rather than single strains has been recently studied and applied more as it may overcome the limitations mentioned above.

Therefore, in this study, we aimed at enriching a microbial community capable of efficiently degrading glyphosate. We also investigated the composition and the diversity of microbial consortia used for the enrichment process in relation to their glyphosate-degrading capabilities.

2. Materials and methods

Microbial samples collected to be used in this study included: (i) a natural soil sample from Cúc Phương National Park (CP), (ii) a natural sediment sample from Vân Long lagoon in Gia Viễn district, Ninh Bình province (VL), (iii) a herbicide-contaminated soil sample from a vegetable field by Hoàng Như Tiếp street, Gia Lâm district, Hanoi (HNT), (iv) a sample soil collected from Đống Đa knoll in Đống Đa district, Hanoi (DD), (v) a soil sample from farm land on the Red river bank in Long Biên district, Hanoi previously treated plant protection chemicals (SH), (vi) a herbicide treated soil sample collected from pine hill in Hữu Lũng district, Lạng Sơn city (LS).

Dilution enrichment procedure: First of all, microbial communities were enriched by using a selective medium. The medium contained: Agar, 16.000 g/L; NaCl, 5.000 g/L;

$K_2PO_4.3H_2O$, 1.470 g/L; KH_2PO_4, 0.480 g/L; $(NH_4)_2SO_4$, 0.132 g/L; $MgSO_4.7H_2O$, 0.246 g/L; Thiamine HCl (100mg/mL), 0.010 mL/L; Glyphosate isopropylamine, 1.000 ml/L. 10 grams of a microbial sample was inoculated into an Erlenmeyer flask containing 90 mL of the enrichment medium and the resulted culture was subsequently incubated while being shaken at 200 rpm at 37 ^{0}C for 24 hours. After that, 10 ml of this liquid culture was subsequently transferred to another Erlenmeyer flask containing 90 mL of the enrichment medium and the resulted solution was subsequently incubated while being shaken under the same conditions for 24 hours. This procedure was repeated 5 times to remove all the irrelevant soil-associated components and selectively enrich the microbial community that can grow on glyphosate.

Isolation of microorganisms: After the enrichment, each sample was diluted to various levels and then cultured on the solid enrichment medium by the plate-spreading method to isolate the microorganisms.

Determination of glyphosate degradation: A 10 ml aliquot from each enrichment sample was transferred to 90 ml of autoclaved enrichment medium and incubated at 200 rpm at 37 ^{0}C. After every 24 hours of incubation, 1 ml of the culture was collected and centrifuged at 3000 rpm, at 25 ^{0}C for 10 min. The pellet (containing cells) was separated and resuspended in 1 mL of the enrichment medium and the OD of this suspension was recorded at 600 nm by an UV-VIS spectrophotometer (Thermo electronic corporation) to measure the cell density. The remaining supernatant was further centrifuged at 14000 rpm, at 25 ^{0}C for 10 min and the pellet was discarded to remove proteins. To quantify the concentration of glyphosate, the obtained supernatant was supplement with ninhydrin (5% w/v) in presence of sodium molybdate (5% w/v) at 100 ^{0}C and the optical absorbance of the final solution was recorded at 570 nm [12].

In-vivo toxicity test: Weeds (*Axonopus compressus*) were dipped into petri plates (at least 3 leaves/plate) containing a sterile minimal medium containing 1µg/ml glyphosate, a sterile minimal medium without glyphosate, or a treated medium. The treated medium was produced by previously growing the selected microbial community in a medium containing 1µg/ml glyphosate in a shaker at 37 ^{0}C and 200 rpm for 7 days.

Conventional methods including plate-streaking method and morphological observations by Gram staining, together with 16S rRNA gene sequencing, were used for identification of microorganisms and assessing the microbial diversity of enriched communities. For 16S rRNA sequencing, total DNA of a single strain or a mixed culture was extracted by using a standard protocol (according to Sambrook & Russell, Molecular Cloning). 16S rRNA gene fragments were amplified by PCR using primers p63F (5'CAGGCCTAACACATGCAAGTC3', forward primer) and p1378 (5'CGGTGTGTACAAGGCCCGGGAACG3', reverse primer) before sequenced by Integrated DNA Technologies (Singapore). After sequencing, the sequences were then edited by CHROMAS version 2.4 and subsequently compared with equivalent 16S rRNA sequences in the database of GenBank by BLAST Search tool.

3. Results

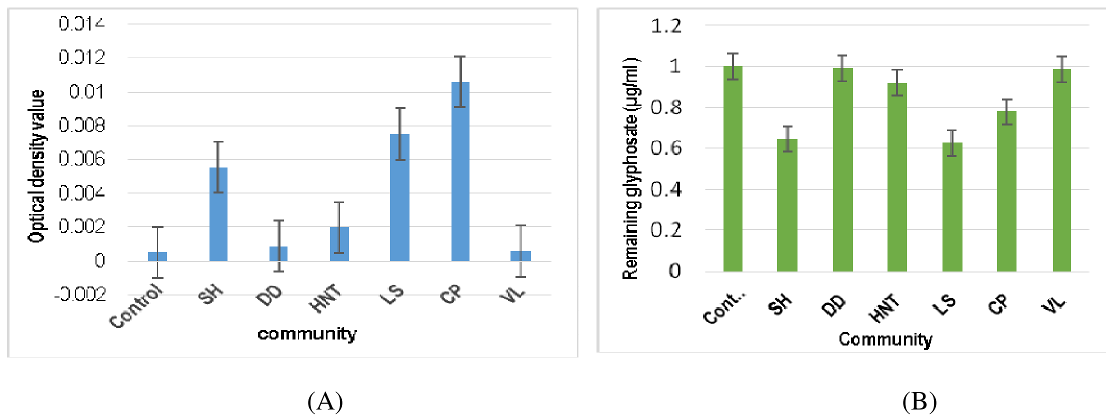
Enrichment of glyphosate-degrading microbial communities by using selective media

All the different microbial samples (CP, VL, HNT, DD, SH, LS) were used as inocula for the enrichment of microbial communities that can degrade glyphosate. After completing the enrichment procedure as described above, the microbial cultures were subjected to centrifugation for the determination of cell growth and residual glyphosate. As can be seen in Fig. 1, three enriched cultures from SH, LS and CP could grow on glyphosate. Their cell densities were significantly higher than those of the other cultures and the control, which was

not inoculated; while the corresponding concentrations of remaining glyphosate were significantly lower.

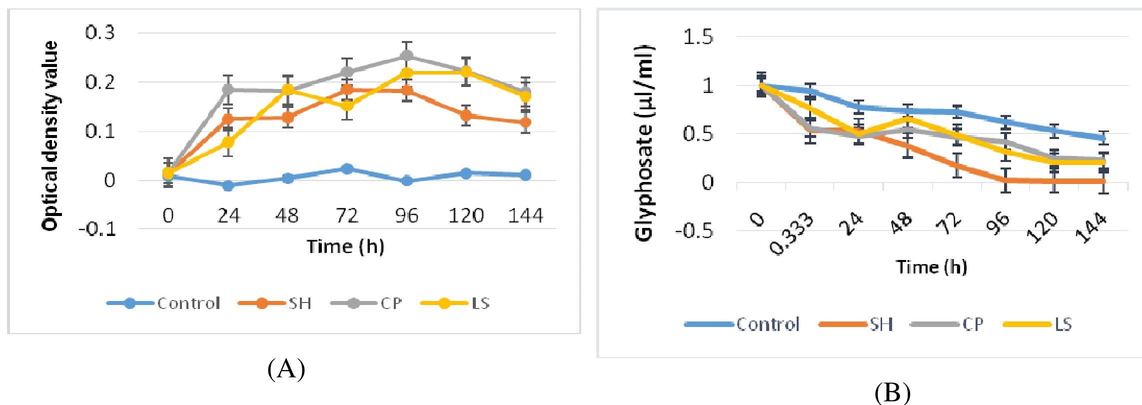
Comparison of the glyphosate-degrading capabilities of the enriched communities

From the results of enrichment process, three enriched cultures from the inocula SH, CP and LS were selected to compare their capabilities of degrading glyphosate. The microbial community in the CP enriched culture gave the best growth (Fig. 2A). However, its glyphosate-degrading ability only ranked the second after that of the SH enriched community (Fig. 2B).



(A) (B)

Figure 1. Cell densities (A) and the concentrations of remaining glyphosate (B) of the enriched cultures afer 24 hours of growth in a glyphosate-containing minimal medium.



(A) (B)

Figure 2. Comparison of three enriched cultures in terms of their cell growths (A) and glyphosate degradations (B).

Toxicity tests of glyphosate-containing solutions treated by a glyphosate-degrading microbial community

The results shown in Fig. 2 suggested that there might be intermediate products that help microbial communities enter a secondary growth and these products might act on glyphosate. Thus it is necessary to test whether glyphosate-containing solutions treated by a glyphosate-degrading microbial community are toxic.

Toxicity tests were carried out (as described in section 2) with glyphosate-containing solutions treated by the SH enriched community, which has the highest glyphosate-treating efficiency. After four days being submerged into the treated glyphosate-containing solutions, the tested weed still appeared similar to that treated with the medium without herbicide (the control) (Fig. 3). This result suggests that the products of the glyphosate biodegradation by the SH enriched community are nontoxic to plants.

Microorganisms in the enriched communities

In order to understand what microbes in the selected microbial communities are and the influences of their composition and diversity on their bioremediation capabilities, microbes of the SH, CP and LS enriched communities were isolated on the solid selective medium and initially investigated by microscopic observation and Gram staining. Strikingly, all the obtained isolates are Gram-negative bacteria, including: four strains from SH community (SH1; SH2; SH3; SH4), three strains from CP community (CP1; CP2; CP3) and three strains from LS community (LS1; LS2; LS3) (Fig. 4).

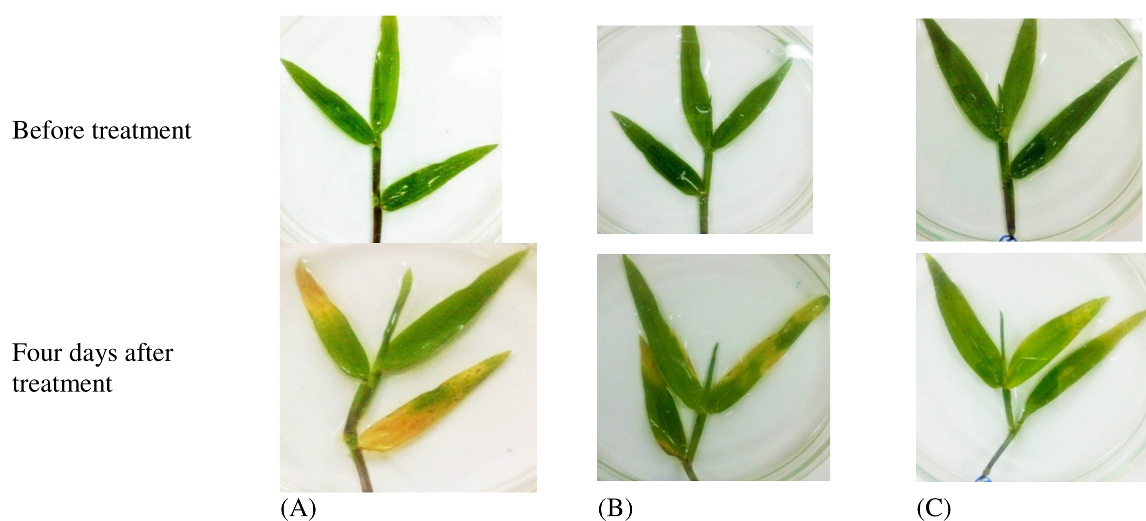


Figure 3. Toxicity tests of glyphosate-containing solutions treated by a SH microbial community.
Notes: (A): samples treated with the medium containing glyphosate (1µg/ml) (positive control); (B): samples treated with only the medium (negative control); (C): samples treated with the medium containing glyphosate (1µg/ml) and previously inoculated with the SH enriched community for 7 days.

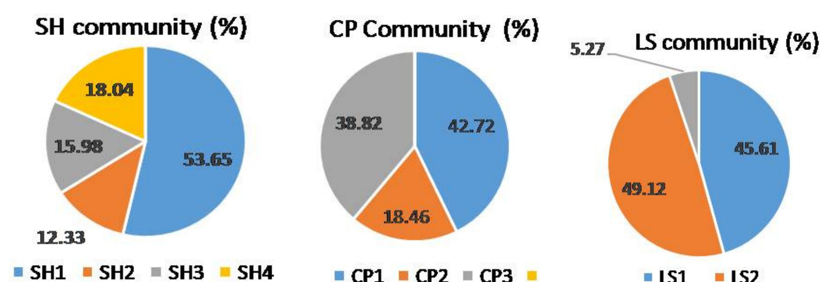


Figure 4. The presence frequences (expressed in percentages) of the microbial isolates from the enriched communities.

The isolates were further identified based on analyzing their 16 rRNA gene sequences. As show in Fig. 5, there are high similarities among those sequences, indicating that the isolates from different communities very much overlap each other. More specifically, SH2 (from the SH enriched community) and CP1 (from the CP one) are probably the same and belong to the genus *Ochrobactrum*. Similarly, SH3 and LS2 might be both a *Enterobacter cloacae* strain; while SH4, CP3 and LS1 might all be a *Pseudomonas* strain. CP2 and LS3 might both belong to the genus *Stenotrophomonas* but are members of different species. A notable difference was the presence of SH1, probably a *Sphingomonas* sp., in only the SH enriched community.

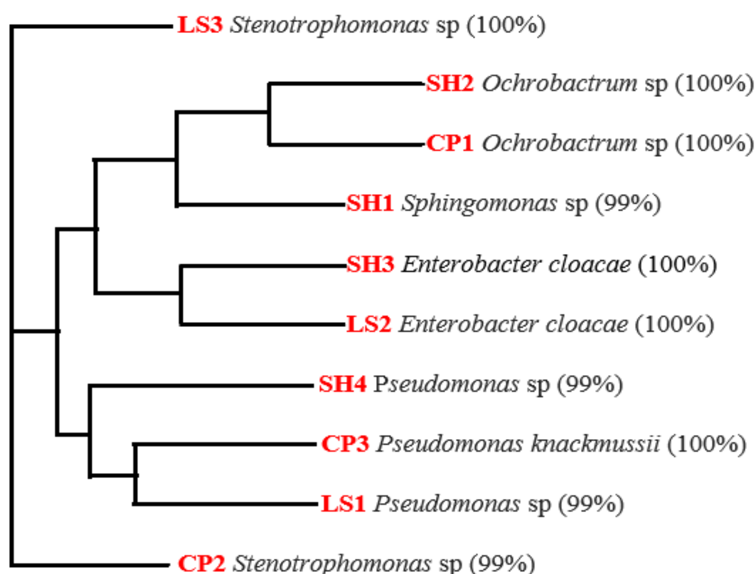


Figure 5. A phylogenetic tree demonstrating the relationships of the isolates in enrichment communities. The tree was created by Neighbor Joining method based on the 16S rRNA gene sequences of the isolates.

Glyphosate degrading capability of the SH community in comparison with those of its individual members:

The glyphosate-degrading capabilities of the SH enriched community and its individual members were compared by growing them in the selective medium containing glyphosate under the same conditions as described and measuring the cell densities and the concentrations of remaining glyphosate in the cultures.

It can be seen from Fig. 6 that, of all single strains of the SH enriched community, the growth rate of SH4 (*Pseudomonas* sp.) was the highest but was not as high as that of the community. Microorganisms in the community entered the stationary phase after 24 hours of inoculation, while for the SH4 strain the stationary phase began approximately after 60 hours (Fig. 6). Notably, glyphosate concentration of the medium inoculated with the community already reduced half after the first 24 hours of inoculation, while for the single strains, this took 48 hours. This illustrated that the community has a higher biodegradation rate than those of its individual strains.

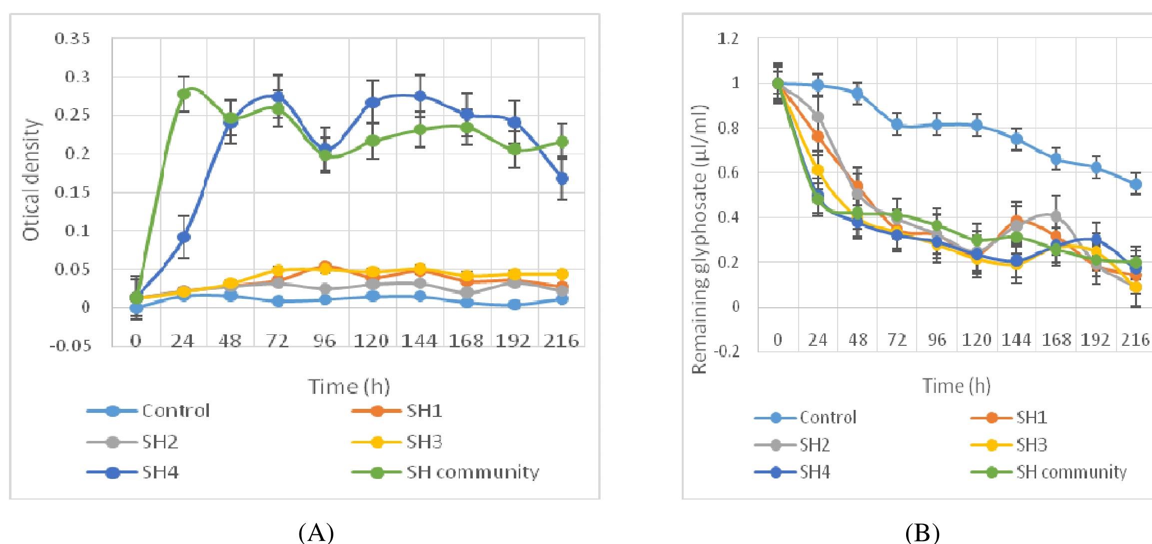


Figure 6. Comparison of the SH enriched community and its individual strains in terms of their cell growths (A) and glyphosate degradations (B).

4. Discussion

The microorganisms from SH, LS and CP samples can survive probably because they can utilize glyphosate, possibly as a carbon source, since the enrichment medium used in this study was plainly a minimal medium containing glyphosate as the only carbon source. In addition, our results (Fig. 4) suggested that the number of isolated strains from enriched communities was not high. A reason for this might be the high selectivity of the enrichment medium. The medium contained only glyphosate functioning as a carbon source, inorganic salts, and water. Thus, mainly microorganisms which can utilize glyphosate could survive.

Although the growth of the CP enriched community on glyphosate seemed to be the best (Fig. 2A), the SH enriched community showed the highest efficiency in degrading glyphosate (Fig. 2B). Hence, there could be some special strains playing an important role in the function of the SH enriched community. These strains might have a crucial ability of utilization of glyphosate so that with a modest population size, they still help the SH enriched community reach the best glyphosate-degrading capacity among all the three selective communities. A reasonable explanation for the more effective degradation of the SH enriched community may be the presence of *Sphingomonas* sp. (strain SH1), since *Sphingomonas* sp. was the only bacterium that makes SH different from the other communities (Fig.5). However, when SH1 was used alone, it glyphosate-degrading capability was not significantly better than the other members (single strains) of the SH enriched community (Fig. 6). This points out that the efficiency of glyphosate bioremediation of the SH enriched community is based on the interactions between *Sphingomonas* sp. with other strains, but not due to only a single strain. The bacterial isolation results by plate-spreading method also support this conclusion. SH1 accounted for 53.65% of the CFUs in the SH enriched community while its growth rate when cultured individually was not high, even lower than that of SH4 (Fig.6).

Our results also indicated that all of three selective communities contained *Pseudomonas* spp. The role of *Pseudomonas* spp. in the biodegradation of glyphosate was also mentioned in previous studies. For example,

Olawale *et al*. reported that *Pseudomonas putida* completely degraded 50 μg/ml glyphosate in 20 ml of an enrichment medium at approximately 72hrs [1, 11]. Therefore, it could be predicted that *Pseudomonas* spp. may play an essential role in the degradation of glyphosate. Probably, the SH enriched community, which could perform better in degrading glyphosate, *Pseudomonas* spp., together with the unique *Sphingomonas* sp. SH1 can enable a more efficient food-web for the consumption of glyphosate.

Altogether our results demonstrated that a mixed culture can function more efficiently than an axenic culture in certain metabolic contexts. Definitely, it was proven above that this better performance is due to the harmonization of single species in the community, which provided added benefits that the axenic cultures cannot have. This leads to a requirement of selecting and preserving well-performing mixed cultures, which is in line with a concept of mixed culture resource management that was proposed previously [13].

5. Conclusion

In this study, we have demonstrated that it is feasible to enriched microbial communities that are capable of efficiently degrading a herbicide and an environmental pollutant such as glyphosate. A microbial community enriched from river bank soil (SH) appeared to perform more efficiently than its single individual members in degrading glyphosate, while causing no harm to plants. A specific community composition and a synergistic community harmonization might be the reason for the better performance of the enriched community, in comparison with the other communities and the single strains. Research on the use of mixed cultures in bioremediation therefore deserves more attentions in the future.

References

[1] J. E. Franz, "N-phosphonomethyl-glycine phytotoxicant compositions", United States Patent Office, 1974.

[2] N. Amrhein et al., "The Site of the inhibition of the shikimate pathway by glyphosate", Plant Physiol. vol 66, pp. 830-834, 1980.

[3] E. Schonbrunn et al., "Interaction of the herbicide glyphosate with its target enzyme 5-enolpyruvylshikimate 3-phosphate synthase in atomic detail", Proceedings of the National Academy of Sciences of the United States of America. vol 98, pp. 1376-1380, 2001.

[4] E. Piesova, "The effect of glyphosate on the frequency of micronuclei in bovine lymphocytes in vitro", Acta veterinaria. vol 2, pp. 101-109, 2005.

[5] UBND tỉnh Quảng Ngãi Tìm thấy chất Glyphosate trong nguồn nước sinh hoạt tại Làng Riềng, http://ubnd.quangngai.gov.vn/quangngai/tiengviet/bangtin/2012/74030/.

[6] S. Chen and Y. Liu, "Study on the photocatalytic degradation of glyphosate by TiO2 photocatalyst", Chemosphere. vol 5, pp. 1010-1017, 2007.

[7] R. Großkopf et al., "Diversity and structure of the methanogenic community in anoxic rice paddy soil microcosms as examined by cultivation and direct 16S rRNA gene sequence retrieval", Applied and Environmental Microbiology. vol 64, pp. 960–969, 1998.

[8] M. M. Higarashia and W. F. Jardim, "Remediation of pesticide contaminated soil using TiO2 mediated by solar light", Catalysis Today. vol 76, pp. 201–207, 2002.

[9] T. M. Balthazor and L. E. Hallas, "Glyphosate-degrading microorganisms from industrial activated sludge", Applied and Environmental Microbiology. vol 51, pp. 432-434, 1986.

[10] D. H. Piepera and W. Reineke, "Engineering Bacteria for bioremediation", Current Opinion in Biotechnology. vol 11, pp. 262-270, 2000.

[11] Olawale et al., "Biodegradation of Glyphosate Pesticide by Bacteria isolated from Agricultural Soil", Report and Oponion. vol 3, pp. 124-128, 2011.

[12] B. L. Bhaskara and P. Nagaraja, "Direct sensitive Spectrophotometric detemination of Glyphosate by using Ninhydrin as a Chromogenic reagent in formulations and enviromental waster samples", View issue *TOC*. vol 89, pp. 2686-2693, 2006.

[13] W. Verstraete et al., "Microbial Resource Management: The Road To Go for Environmental Biotechnology", View issue *TOC*. vol 7, pp. 117-126, 2007.

Nghiên cứu làm giàu và chọn lọc quần xã vi sinh vật có khả năng phân giải thuốc diệt cỏ glyphosate

Hoàng Thị Oánh, Phạm Thế Hải

Trường Đại học Khoa học Tự nhiên, ĐHQGHN, 334 Nguyễn Trãi, Hà Nội, Việt Nam

Tóm tắt: Diệt trừ và khống chế sự phát triển của cỏ dại là công việc bắt buộc trong canh tác nông nghiệp. Glyphosate là một trong những hoạt chất diệt cỏ được sử dụng rộng rãi nhất trên toàn thế giới. Tuy nhiên, đây cũng là một chất ô nhiễm môi trường với dư lượng ở Việt Nam và trên thế giới đang ở mức rất báo động. Hiện nay, các phương pháp xử lý glyphosate, bao gồm vật lý, hóa học và sử dụng các đơn chủng vi sinh vật còn gặp rất nhiều hạn chế. Do đó, chúng tôi thực hiện nghiên cứu này với mục đích tìm ra các quần xã vi sinh vật có khả năng phân giải hiệu quả glyphosate. Các vi sinh vật từ các mẫu khác nhau được làm giàu bằng phương pháp pha loãng trên môi trường nuôi cấy chọn lọc có chứa glyphosate. Ba quần xã vi sinh vật có khả năng phân giải glyphosate đã được làm giàu thành công: SH, CP và LS. Dung dịch môi trường có chứa hoạt chất diệt cỏ glyphosate sau khi được xử lý bởi quần xã vi sinh vật SH đã được chứng minh là không còn tính độc đối với thực vật. Kết quả so sánh khả năng phân giải hoạt chất diệt cỏ của quần xã SH với các đơn chủng của quần xã (bao gồm *Sphingomonas* sp (SH1), *Ochrobactrum* sp (SH2), *Enterobacter cloacae* (SH3)và *Pseudomonas* sp (SH4)) cho thấy trong cùng một điều kiện nuôi cấy, quần xã SH có tốc độ phân giải tốt hơn các đơn chủng. Có lẽ cấu trúc quần xã đặc trưng kết hợp với mối quan hệ hỗ trợ của các chủng vi sinh vật có trong quần xã là nguyên nhân chính khiến quần xã SH có hiệu suất xử lý hoạt chất diệt cỏ tốt hơn các đơn chủng và các quần xã khác. Kết quả của nghiên cứu này cho thấy việc nghiên cứu và sử dụng các quần xã vi sinh vật (thay vì các đơn chủng) trong công tác hồi phục sinh học là cần thiết và do đó xứng đáng nhận được nhiều sự quan tâm hơn nữa của các nhà khoa học.

Từ khóa: Sự phân giải, thuốc diệt cỏ, glyphosate, quần xã vi sinh vật, phục hồi sinh học, *Sphingomonas* sp., *Pseudomonas* sp.